GLYPHOSATE RESISTANCE IN KOCHIA

by

AMAR SINGH GODAR

B.S., Tribhuvan University, 2003
M.S., Kansas State University, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Agronomy
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

Abstract

Kochia [*Kochia scoparia* (L.) Schrad.] is a weed of great economic importance in the Great Plains and western United States and Canada. This weed is prone to evolving resistance to herbicides. Glyphosate is the most widely used herbicide in glyphosate-resistant crops and chemical fallow, and is extremely valuable to crop production. Anecdotal reports of kochia control failure with glyphosate in western Kansas arose during the mid-2000's. The objectives of this research were to (1) confirm and characterize glyphosate resistance in kochia and measure its impact in western Kansas, (2) gather information on grower weed management practices before and since glyphosate resistance in kochia was confirmed, and (3) determine if altered absorption and translocation of glyphosate contributes to glyphosate resistance in kochia. Dose-response studies on greenhouse and outdoor grown plants, and shikimate accumulation assays confirmed one kochia population collected in 2007 and eight populations collected in 2010 tolerated three- to eleven-times more glyphosate compared to a known glyphosate-susceptible (GS) population. Furthermore, 40 kochia populations collected in 2012 showed varied response, from slightly elevated tolerance to resistance to 0.84 kg ae ha^{-1} glyphosate. Further analysis suggested these populations were at different stages of resistance evolution. An online survey revealed that growers increased glyphosate use rate and application frequency, but decreased exclusive use of glyphosate and diversified weed management practices during post- compared to pre-glyphosate confirmation periods. Most survey respondents reported presence of glyphosate-resistant (GR) kochia in at least in few fields, and half reported GR kochia in a majority of fields. Thus, together with the resistance confirmation studies, it is estimated that at least one-third of western Kansas kochia populations have evolved resistance to glyphosate. Nominal differences in absorption and translocation of ^{14}C-glyphosate observed between GS and GR kochia populations likely do not contribute to differential response of these populations to glyphosate. Glyphosate-resistant kochia has become widespread in western Kansas in a short period of time. Use of weed resistance best management practices (BMP) is imperative to sustain the utility of glyphosate in the region.

GLYPHOSATE RESISTANCE IN KOCHIA

by

AMAR SINGH GODAR

B.S., Tribhuvan University, 2003
M.S., Kansas State University, 2009

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Agronomy
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

Approved by:

Major Professor
Phillip W. Stahlman

Copyright

Abstract

Kochia [*Kochia scoparia* (L.) Schrad.] is a weed of great economic importance in the Great Plains and western United States and Canada. This weed is prone to evolving resistance to herbicides. Glyphosate is the most widely used herbicide in glyphosate-resistant crops and chemical fallow, and is extremely valuable to crop production. Anecdotal reports of kochia control failure with glyphosate in western Kansas arose during the mid-2000's. The objectives of this research were to (1) confirm and characterize glyphosate resistance in kochia and measure its impact in western Kansas, (2) gather information on grower weed management practices before and since glyphosate resistance in kochia was confirmed, and (3) determine if altered absorption and translocation of glyphosate contributes to glyphosate resistance in kochia. Dose-response studies on greenhouse and outdoor grown plants, and shikimate accumulation assays confirmed one kochia population collected in 2007 and eight populations collected in 2010 tolerated three- to eleven-times more glyphosate compared to a known glyphosate-susceptible (GS) population. Furthermore, 40 kochia populations collected in 2012 showed varied response, from slightly elevated tolerance to resistance to 0.84 kg ae ha^{-1} glyphosate. Further analysis suggested these populations were at different stages of resistance evolution. An online survey revealed that growers increased glyphosate use rate and application frequency, but decreased exclusive use of glyphosate and diversified weed management practices during post- compared to pre-glyphosate confirmation periods. Most survey respondents reported presence of glyphosate-resistant (GR) kochia in at least in few fields, and half reported GR kochia in a majority of fields. Thus, together with the resistance confirmation studies, it is estimated that at least one-third of western Kansas kochia populations have evolved resistance to glyphosate. Nominal differences in absorption and translocation of ^{14}C-glyphosate observed between GS and GR kochia populations likely do not contribute to differential response of these populations to glyphosate. Glyphosate-resistant kochia has become widespread in western Kansas in a short period of time. Use of weed resistance best management practices (BMP) is imperative to sustain the utility of glyphosate in the region.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I am heartily thankful to my major advisor, Dr. Phillip W. Stahlman. His supervision, support, and encouragement from the preliminary to the concluding level qualified me to successfully complete my doctoral program.

I also thank Dr. J. Anita Dille, for serving as my academic advisor as well as graduate committee member. She was always there to meet and talk about my ideas and problems, to proofread and mark up my writings, posters, and presentations. I would like to extend my thanks to Dr. Mithila Jugulam for her guidance in crucial parts of my dissertation research, serving on my graduate committee, and providing valuable advice. Appreciation extends to Dr. Richard B. Todd for serving in my graduate committee and to Dr. Anthony Joern for chairing the final examination.

Let me also say ‘thank you’ to Patrick Geier for supporting my research activities at Kansas State University Agricultural Research Center, Hays, KS. I would also like to give special thanks to Linda Stahlman for her love and support while being in Hays during the summer months.

Thanks to fellow graduate students at KSU Agronomy Department for their friendship, assistance, and input into my research.

I thank my parents, Hirakul Godar and Bel Kumari Godar for unconditional support and encouragement to pursue my interests, even when the interests went beyond boundaries of language and geography. I would also like to thank my wife for her support, patience, and encouragement.

Last, but not least, I offer my regards and blessings to all of those who supported me in any respect during the completion of this program.

Dedication

This dissertation is dedicated to my parents, Hirakul and Bel Kumari, and my lovely wife, Puja. Thank you all for your love, support, and patience.

Chapter 1 - Confirmed Glyphosate Resistance in Kochia in Western Kansas

Abstract

Seeds from kochia plants suspected of resistance to glyphosate were collected from a glyphosate-resistant (GR) soybean field in Thomas County, KS in 2007 (2007 collection) and from eight additional fields throughout western Kansas in 2010 (2010 collection) to confirm and characterize glyphosate resistance in those populations. A known glyphosate-susceptible (GS) kochia population in Ellis County KS was used for comparison in all studies. Most plants from the 2007 collection survived multiple applications of glyphosate from 0.56 up to 1.12 kg ae ha^{-1} under outdoor conditions and produced viable seeds. Several 2nd generation progeny plants survived up to 1.68 kg ae ha^{-1} glyphosate under greenhouse conditions and up to 6.72 kg ae ha^{-1} under outdoor conditions. In whole plant bioassay (2010 collection) under greenhouse and/or outdoor environments, a series of glyphosate rates ranging from 0 to 6.72 kg ae ha^{-1} were applied, and mortality and aboveground dry biomass were determined 3 to 4 weeks after glyphosate treatment. Based on the ED_{50} (the dose causing 50% mortality) and GR_{50} (the dose causing 50% biomass reduction) values, the suspected GR kochia populations were three- to eleven-times more resistant to glyphosate compared to the GS population. An *in vivo* shikimate assay was performed on eight plants from each population (2010 collection) by treating 4-mm leaf discs with 100 µM glyphosate and incubating for 16 h under continuous light. The GS population accumulated consistently higher amounts of shikimate (148 to 227 ɳg mm^{-2} leaf disc) than the suspected GR populations (5.25 to 116 ŋg mm^{-2} leaf disc). This study confirmed the 2007 Thomas County population and eight additional populations collected in 2010 were resistant to glyphosate, hence, indicating widespread presence of GR kochia in western Kansas.

Introduction

Kochia [*Kochia scoparia* (L.) Schrad.] is an annual broadleaf species introduced to North America from Eurasia in the 1890's (Durham and Durham 1979). This alien species naturalized and became well established in semiarid and arid regions of the Great Plains (Everitt et al. 1983). It subsequently spread throughout the west-central and northwestern United States (Forcella 1985) and into the Canadian prairies (Friesen et al. 2009) where it is commonly found in cropland and noncrop areas, such as pastures, disturbed natural areas, roadsides, and railroad rights-of-way. Ecological attributes such as rapid seed germination in early spring at low temperatures (Anderson and Nielsen 1996, Anderson 1994, Dille et al. 2012) and ability to tolerate drought, heat, and salinity contribute to its rapid spread (Forcella 1985).

Kochia is a troublesome weed of economic importance in crop production systems (Dexter et al. 1981, Friesen et al. 2009, Wicks et al. 1984). While early emerged kochia have distinct survival and competitive advantages in cropping systems (Evetts and Burnside 1972), late emerging plants may escape POST herbicide applications (Leeson et al. 2005, Mickelson et al. 2004, Mulugeta 1991), resulting in seed production and seedbank renewal (Schwinghamer and Van Acker 2008). In addition, kochia is well-adapted to prolonged dry conditions (Eberlein and Fore 1984, Kocacinar and Sage 2003) that often occur during summer months in the Central Great Plains. Precipitation in western Kansas (west of Hwy 81) decreases from about 750 to 350 mm annually from east to west (Keim 2010). Kochia abundance generally is greater in the drier region of the state. Kochia can cause severe yield loss in several crops (Dahl et al. 1982, Durgan et al. 1990, Wicks et al. 1994), however, the major concern is its presence in winter wheat stubble or summer fallow fields in the region, especially in no-till systems.

Weed populations are often reduced in no-till systems because of less soil disturbance and increased accumulation of crop residues (Anderson 2004, Crutchfield et al. 1986). Conversely, such practices can increase kochia emergence by almost four-fold compared to tilled systems (Anderson and Nielsen 1996). Thus, transformation of cropping practices from conventional tillage to no-till in the past few decades has favored seedbank dynamics of kochia, resulting in its greater abundance in the region. Tillage not only effectively controls kochia plants but also depletes the seedbank by burying seeds deeper in soil than seedlings can emerge.

Glyphosate is a major weed management component in Great Plains cropping systems. Duke and Powles (2008) described glyphosate as a ‘once-in-a-century’ herbicide. The rapid adoption of Roundup Ready® crops coupled with the exceptionally high efficacy and declining cost of glyphosate attracted growers to use glyphosate extensively, often exclusively, for controlling weeds in crop production systems. Also, no-till systems require use of herbicides to control weeds during fallow periods between crops and prior to planting. Glyphosate is the herbicide most often used in both instances. Thus, the dramatic success of glyphosate-resistant crops and increased use of no-till crop production systems have intensified the use of glyphosate in the U.S. during the past two decades.

Until the 1990’s winter wheat-fallow was the predominant cropping system in far western Kansas (Eberle and Shroyer 1997). Though winter wheat continues to be a major component of the cropping systems in the region, rotations have become more diversified. Winter wheat was planted on 39% of total cropland (5.46 million ha) in western Kansas in 2012 (Han et al. 2012). Several selective herbicides with diversified modes of action may be applied for growing season weed control in winter wheat. Kochia management in standing wheat is often challenging because of the presence of kochia biotypes resistant to commonly applied herbicides such as triazines and acetolactate synthase (ALS)-inhibitors, thus, often requiring post-harvest herbicide applications (Mickelson et al. 2004). Cropping systems in western Kansas have shifted from winter wheat-fallow to winter wheat-row crop-fallow rotations, which usually includes corn (19%), grain sorghum (12%), soybean (2.8%), sunflower (0.12%), or cotton (0.08%). Corn, soybean, and cotton are mostly glyphosate-resistant (GR) varieties. In 2012, 23% of total cropland was left fallow or idle (Han et al. 2012). During the late 1990's and early 2000's, glyphosate was the most commonly-used herbicide for controlling summer annual weeds including kochia in fallow (including post-wheat harvest) and was used almost exclusively in GR crops in the region. These use patterns contributed to increased selection pressure for resistance to glyphosate.

Kochia possesses many characteristics that enhance response to selection and subsequent spread of selected traits. For example, high emergence of kochia in no-till systems, high genetic variability (Mengistu and Messersmith 2002), and short seed life (Schwinghamer and Van Acker 2008, Thompson et al. 1994) promote rapid response to selection pressure. Protogynous flowering (Stallings et al. 1995) in kochia facilitates open pollination, which, in turn, also favors exchange of selected traits. Furthermore, the tumbling architecture of mature kochia plants (Fay et al. 1992) enhances seed dispersal and rapid spread of selected traits over long distances (Jasieniuk and Maxwell 1994). Historically, kochia has been prone to evolution of resistance to herbicides such as resistance to triazines in 1976, ALS-inhibiting herbicides in 1986, and dicamba in 1995 (Heap 2013, Foes et al. 1999, Peterson 1999). Kochia biotypes resistant to triazines and ALS-inhibiting herbicides were reported first in Kansas (Heap 2013). While several other GR weeds have been confirmed in Great Plains states (Heap 2013), those weeds are not common in the semiarid winter wheat growing region.

In 2007, a trail of kochia plants across an irrigated GR soybean field near Colby (Thomas Co.), KS was observed (Figure 1.1). The field had been sprayed with glyphosate three times after soybean emergence. The dispersal plant came from an adjacent GR corn field. Several additional cases of kochia control failure were reported in western Kansas during the following years. The objectives of this study were to (1) determine if the kochia population from Thomas Co., KS (2007 collection) was resistant to glyphosate, (2) confirm and characterize eight additional geographically dispersed populations (2010 collection) in western Kansas, and (3) compare the response of GR kochia to glyphosate under greenhouse and outdoor environments.

Materials and Methods

Seed Collection and Preliminary Screening

Surface soil was collected in spring 2008 from a field near Colby, KS (Figure 1.1) where the previous fall suspected GR kochia plants were pulled or cut at the base of the plant, piled and burned. Soil from the burn area was placed in metal trays in a greenhouse and surface watered. Two hundred sixty-five seedlings were transplanted individually into 1.6 L pots filled with soil (Roxbury silt loam with 1.5% organic matter and pH 7.8). A few days after transplanting, the pots were submerged to within 3 cm of the rim in a field at the Kansas State University Agricultural Research Center at Hays, KS, where the plants continued growing under natural environmental conditions. Plants were sprayed three different times with increasing rates of glyphosate (Roundup Weathermax®, Monsanto Company, St. Louis, MO) using a tractor-mounted, compressed-air sprayer delivering 120 L ha^{-1} spray volume and evaluated visually after each application (Table 1.1). Surviving plants were returned to semi-controlled greenhouse conditions prior to flowering (late summer) and were allowed to cross pollinate. Plants were grown under 15/9 h day/night photoperiod, supplemented with 120 µmol $m^{-2}s^{-1}$ illumination provided with sodium vapor lamps. Seed were collected from individual (parental) plants for further testing. Progeny from several parental plants were grown in separate plastic trays and 2-cm tall seedlings were transplanted into soil-filled pots and placed in a greenhouse (0.8 L pot) or outdoors (1.6 L clay pot) during summer 2009. Plants were sprayed when 10- to 15-cm tall with four rates of glyphosate (0.84, 1.68, 3.36, and 6.72 kg ae ha^{-1}) and ammonium sulfate (AMS, 2% w/v). A known GS population from Ellis Co., KS (Ellis hereafter) was grown similarly for comparison. Glyphosate was applied with a moving single-nozzle bench-type sprayer (Research Track Sprayer, De Vries Manufacturing, RR 1 Box 184, Hollandale, MN) equipped with a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., P.O. Box 7900, Wheaton, IL) delivering 168 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. Treatments were replicated three times and the experiment was not repeated. Mortality was evaluated 2 weeks after treatment (WAT).

In fall 2010, seed from 17 additional populations from cropland fields with unknown cropping history were collected in western Kansas. Seed from most sites were harvested from more than 10 mature plants per site and bulked in separate bags for each population. Twelve progeny plants from each population were grown individually in 0.8 L pots in a greenhouse and were treated with a 0.84 kg ae ha^{-1} glyphosate and 2% w/v AMS using the single-nozzle bench sprayer described above. Mortality and injury levels were evaluated 2 WAT.

Whole Plant Dose-response Assay on Eight Prescreened Populations

Based on results of preliminary studies with 2010 seed collections, the following eight populations, Hodgeman, Lane101, Lane102, Norton, Phillips, Russell, Scott99, and Scott100 were selected for a whole plant glyphosate dose-response assay. Geographic locations of the populations are shown in Figure 1.2.

This experiment was conducted in July-August, 2011 at Hays, KS in a semi-controlled greenhouse environment. Plants were grown in 15/9 h day/night photoperiod, supplemented with 120 µmol $m^{-2}s^{-1}$ illumination provided with sodium vapor lamps. Temperature was not fully controlled, so indoor temperature was 2 to 4 C higher during mid- and late-afternoon compared to outdoor temperature. Plants were treated using the same procedure described previously. Glyphosate doses for the eight suspected GR populations were 0, 0.17, 0.34, 0.68, 1.36, 2.72, and 5.44 kg ae ha^{-1}, and 0, 0.085, 0.17, 0.34, 0.68, 1.36, and 2.72 kg ae ha^{-1} for the GS Ellis population. All treatments included 2% w/v AMS. The experiment was arranged in a completely randomized design with six replications per treatment. Mortality and aboveground dry biomass were recorded at 3 WAT.

Whole Plant Dose-response Analysis under Greenhouse and Outdoor Environments

Of the eight selected prescreened populations, Phillips, Scott100, and known GS Ellis populations were selected for dose-response analysis under greenhouse and outdoor conditions during June-July 2012 at Hays, KS. Greenhouse plants were grown in 0.8 L black plastic pots and outdoor plants were grown in 1.6 L clay pots. Temperatures under greenhouse conditions were 25/20 C d/n with no supplemental lighting, and plants were watered as needed. However, outdoor plants sometimes exhibited moderate moisture stress as plants experienced greater temperature fluctuation and evapotranspiration than greenhouse-grown plants. Glyphosate was applied to 15- to 20-cm tall plants using the single-nozzle bench sprayer described previously. Plants were returned to their respective environments immediately after spraying. Glyphosate doses for Phillips and Scott100 populations were 0, 0.42, 0.84, 1.68, 3.36, and 6.72 kg ae ha^{-1} and 0, 0.21, 0.42, 0.84, 1.68, and 3.36 kg ae ha^{-1} for the GS Ellis population. The experiment included six replications and was repeated. The treatments were arranged in a completely randomized design. At 4 WAT, mortality and injury symptoms were recorded and plants were harvested for dry biomass as described above.

Shikimate Accumulation Assay

Before applying glyphosate in whole plant dose-response assay, eight plants from each of the nine populations (eight suspected GR and one known GS) were selected randomly for *In-vivo* leaf disc shikimate accumulation assay using the procedure described by Shaner et al. (2005). Eight 4-mm leaf discs (in quadruplet; four treated and four controls) were excised from the two youngest fully-expanded leaves from each plant. Four leaf discs from each plant were placed in transparent 96-well microtiter plates containing 100 µL of 100 µM glyphosate (Glyphosate PESTANAL®, analytical standard, Sigma-Aldrich Co. LLC, St. Louis, MO) solution and another four leaf discs were placed in 100 µL buffer (Shaner et al. 2005) without glyphosate. The microtiter plates were wrapped with clear plastic to reduce evaporation and incubated in a growth chamber at 25 C for 16 h under continuous light (200 mmol m^{-2}s^{-1}). The plates were frozen at -20 C for 20 min and thawed at 60 C for 20 min. Twenty-five microliters of 1.25 N HCl was added to each well, and plates were covered and incubated at 60 C for 25 min. The extracted shikimate in the wells was oxidized by transferring 25-µL aliquot from each well into corresponding wells of new plates containing 100 µL 0.25% periodic acid/0.25% sodium-*m*-periodate solution and incubating for 20 min at 40 C. The same volume of known concentrations of shikimate solution was also transferred similarly to generate a standard curve. After oxidation, the solution was quenched by adding 100 µL 0.6 M NaOH/0.22 M Na_2SO_3 to each well. Optical density was measured immediately using a spectrophotometer (Epoch Micro-Volume Spectrophotometer System, BioTek, Winooski, VT) at 380 nm. Optical density (OD_{380}) values were converted to shikimate concentration (ng μL^{-1}) by using the shikimate standard curve. The amount of shikimate extracted was calculated by subtracting the amount extracted in corresponding control (0 µM glyphosate) wells. The ng μL^{-1} shikimate values were further converted to ng shikimate mm^{-2} of leaf discs (ng mm^{-2} leaf disc). The experiment was done in quadruplets (four controls and four treated leaf discs for each plant).

Single Discriminating Dose on Selfed Progenies

Two to four individual plants from the Ellis, Lane101, and Phillips populations were grown in a greenhouse and individually covered with pollination bags before flowering. Seed from each plant was harvested separately. The progeny seeds were grown in individual 5-cm plastic cones containing commercial potting mix. Plants were treated with 0.84 kg ae ha^{-1} glyphosate with 2% w/v AMS when plants were 10 cm tall. Plant survival was assessed visually at 2 WAT. Plants with no visible green tissue were considered dead.

Statistical Analysis

Data from dose-response studies were analyzed using a nonlinear regression model (Seefeldt et al. 1995). Multiple dose-response models were analyzed and compared with one-way ANOVA by a lack-of-fit F-test using R 2.15.2 (Knezevic, et al. 2007, R Development Core Team 2013, Ritz and Streibig 2005). The three-parameter non-linear log-logistic model (Equations 1.1) showed good fit, thus, the relationship between herbicide dose and mortality or aboveground biomass was described as

$$Y = \frac{d}{\{1 + \exp(b(\log(x) - \log(e)))\}} \qquad \text{Eq. 1.1}$$

where Y is mortality (%) or aboveground biomass, *e* (also known as ED_{50} or GR_{50}) denotes the herbicide dose that caused 50% response, *d* is the response upper limit, *b* denotes the relative slope around *e*, and x represents herbicide dose. The response lower limit was set equal to 0. Additional parameters ED_{10} and ED_{90} (herbicide doses that caused 10 and 90% mortality, respectively) were calculated to determine range of tolerance in individual plants in the populations. Resistance index (RI) was calculated as:

$$\text{Resistance index (RI)} = \frac{ED_{50}\text{ (p)}}{ED_{50}\text{ (susceptible control)}} \qquad \text{Eq. 1.2}$$

where ED_{50} is from Equation 1.1 and p is the population in question.

Shikimate accumulation in plants from GS and suspected GR kochia populations were analyzed using one-way ANOVA in R (R Development Core Team 2013) and the means were compared using Fisher's protected LSD at $\alpha = 0.05$.

Results

Preliminary Screening

Only seven of 265 plants from the 2007 collection were visibly unaffected, whereas 249 were injured but recovered, and nine died following an application of 0.56 kg ae ha^{-1} of glyphosate (Table 1.1). Subsequently, 223 of 256 plants survived two additional applications of glyphosate at 0.84 followed by 1.12 kg ae ha^{-1} and produced viable seed. Plant tolerance to glyphosate among progeny from the same and different parental plants varied widely, but tolerance generally decreased with increasing glyphosate use rate (data not shown). Most progeny recovered from injury (varying levels of injury among the plants) caused by 1.68 kg ae ha^{-1} glyphosate and produced seed. Several outdoor-grown progeny survived 3.36 kg ae ha^{-1} of glyphosate and a few survived 6.72 kg ae ha^{-1}. However, no greenhouse-grown progeny survived more than 1.68 kg ae ha^{-1} of glyphosate (data not shown).

Most kochia plants of Hodgeman, Lane101, Lane102, Norton, Phillips, Russell, Scott99, and Scott100 populations survived 0.84 kg ae ha^{-1} glyphosate at 3 WAT (data now shown). Injury symptoms on surviving plants varied at 3 WAT, but none of the plants were injured more than 50% (0% = visibly unaffected and 100% = dead). Plants of the other nine populations were injured >75%; most were killed. The frequency of individual plants exhibiting ‘‘slightly-elevated tolerance’’ to glyphosate was very low (<10%), thus those populations were not tested further.

Whole Plant Dose-response Assay on Eight Prescreened Populations

The three parameter log-logistic model fit the data across the glyphosate doses for mortality and aboveground biomass (Figure 1.3).

Dose-response curves for mortality 3 WAT are shown in Figure 1.3a. Slope of the curves (*b*) varied widely among populations (-2 to -19 with -11 for the Ellis population). ED_{50} for the Ellis population was 0.17 kg ae ha^{-1} and the values for all suspected GR populations were greater (0.54 to 1.35 kg ae ha^{-1}) (Table 1.2). Among the eight suspected GR populations, Lane102 had the greatest and Norton had the lowest ED_{50} values. With exception of the Norton population, ED_{50} values were greater for populations with larger *b* values. ED_{90} for Ellis and ED_{10} for suspected GR populations did not differ despite large differences between mean values. Resistance index values for Norton, Lane101, Hodgeman, and Phillips populations ranged from

3.3 to 4.5, and the values for Russell, Scott100, Scott99, and Lane102 populations ranged from 6.3 to 8. The ED_{90} value for Ellis was 0.21 kg ae ha^{-1} and 0.77 kg ae ha^{-1} (Lane101) to 3.10 (Scott100) for the suspected GR populations.

Dose-response curves for aboveground dry biomass 3 WAT are shown in Figure 1.3b. Variability in slopes of the curves (*b*) was observed among the populations (1.7 to 5.5), however the slopes for most populations were much lower compared to those for mortality curves (Table 1.3), indicating survival with some injury. GR_{50} value for the Ellis population (0.09 kg ae ha^{-1}) was lower than all the suspected GR populations (0.41 to 0.95 kg ae ha^{-1}). Resistance index values for suspected populations ranged from 4.7 (Russell) to 10.8 (Scott99) kg ae ha^{-1}. Except for Scott99 (GR_{10} = 0.64 kg ae ha^{-1}), GR_{90} for the Ellis population (0.23 kg ae ha^{-1}) did not differ from the GR_{10} for other populations.

Whole Plant Dose-response Assay under Greenhouse and Outdoor Environments

Individual plants of Ellis and Scott100 populations 8 d after treatment are shown in Figure 1.4. The three parameter log-logistic model fit the data for dose-response curves for mortality and aboveground biomass for the Ellis, Phillips, and Scott100 populations (Figure 1.5).

Dose-response curves for mortality 4 WAT are shown in Figure 1.5a. Slope of the curves (*b*) for both environments was steeper for the Ellis population (-4.8 to -11.7) compared to Phillips and Scott100 populations (-3.4 to -3.5) (Table 1.4). For both environments, ED_{50} values for Phillips and Scott100 were higher compared to the Ellis population. The values were 1.5- to 1.9-times higher for outdoor-grown plants compared to greenhouse-grown plants. However, RI values were similar for both environments, and ranged from 3.3 to 4.1 for the Phillips and 5.0 to 5.1 for the Scott100 population. Steeper slope of the curves (*b*) for the Ellis population in the outdoor environment resulted in much greater differences in ED_{90} for Phillips (5.2-fold) and Scott100 (7.8-fold) relative to the Ellis population. ED_{90} value for the Ellis population and ED_{10} value for the Phillips and Scott100 populations did not differ significantly.

Dose-response curves for aboveground dry biomass 4 WAT are shown in Figure 1.5b. Parameter estimates for *b* were greater for all the three populations grown outdoors (3.4 to 5.3) compared to greenhouse environment (2.7 to 3.8) (Table 1.5), suggesting greater individual variation in outdoor conditions. GR_{50} values for Phillips and Scott100 populations were greater compared to the GR_{50} of the Ellis population under both environments. However, for both Phillips and the Scott100 populations the RI values did not differ between environments and were similar to those for mortality. The GR_{90} value for the Ellis population differed from the GR_{10} for the Phillips and Scott100 populations only under greenhouse environments.

Shikimate Accumulation in Leaf Discs

Box and whisker plots (n = 8 plants per population) showing shikimate accumulation in leaf discs in nine populations are shown in Figure 1.6. In all populations, shikimate accumulation varied among individuals within a population as evidenced by the length of box and whiskers (values for individual plants not shown). All individual plants of the Ellis population accumulated more shikimate (148 to 227 ng mm^{-2} leaf disc) compared to individual plants of suspected GR populations (5.25 to 116 ng mm^{-2} leaf disc) ($p<0.001$). Most plants from the Ellis population accumulated 160 ng mm^{-2} leaf disc or more shikimate. In comparison, most plants from suspected GR populations accumulated 80 ng mm^{-2} leaf disc or less shikimate. On average, the suspected GR populations accumulated 2.6- to 6.5-times less shikimate compared to the Ellis population.

Single Discriminating Dose on Selfed Progenies

None of the selfed progeny plants from the Ellis population survived 0.84 kg ae ha^{-1} glyphosate 2 WAT (Table 1.6). All plants from the Phillips and 86% of selfed progeny plants from the Lane101 population survived glyphosate treatment 2 WAT.

Discussion

Several first and second generation progeny of the 2007 Thomas Co. population survived 0.84 kg ae ha^{-1} or greater rates of glyphosate under greenhouse and outdoor conditions, hence confirming the presence of GR kochia in Kansas. However, this population was not characterized for level of resistance. The primary purpose of whole plant dose-response analysis was to confirm and characterize additional GR kochia populations collected in fall 2010. Whole plant dose-response assays confirmed that all eight selected kochia populations were resistant to glyphosate. Waite et al. (2013) reported increased tolerance in three other kochia populations from western Kansas. Together, these provide conclusive evidence of widespread presence of GR kochia in western Kansas. Based on ED_{50} and GR_{50} values, level of resistance in the GR populations ranged from 3 to 11. Similar levels of resistance in several other GR weed species such as Palmer amaranth, Italian ryegrass, hairy fleabane, and horseweed have been documented (Culpepper et al. 2006, Norsworthy et al. 2008, Perez-Jones et al. 2005, Urbano et al. 2007, VanGessel 2001).

In addition to the most commonly used parameter ED_{50} or $GR_{50,}$ knowing the 10% (ED_{10} or GR_{10}) and 90% (ED_{90} or GR_{90}) response values also helps better characterize herbicide resistant populations. As noted by Seefeldt et al. (1995), these estimates were subject to greater errors. Comparison of ED_{10} values for the resistant populations to ED_{90} values for the susceptible population help conclude the presence or absence of susceptible individuals in the resistant populations. Nevertheless, ED_{50} is the function of both the level of resistance in individual plants and frequency of resistant individuals in the population.

Wide variability in tolerance to glyphosate doses within populations was observed, especially in populations with less steeper slopes (*b* values). Interestingly, susceptible plants, if present in a GR population, survived ED_{50} dose for the susceptible population. This may indicate an elevated level of tolerance to glyphosate in all individuals in GR populations. The mechanism of glyphosate resistance in the kochia populations from the Central Great Plains is thought to be due to amplified 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS) genomic copy number (relative EPSPS:ALS genomic copy number = 3 to 9) (Wiersma 2012). This is the same mechanism that was first reported in GR Palmer amaranth (Gaines et al. 2010). Presence of three copies of EPSPS gene relative to ALS gene copy number confers adequate resistance to normal field use rate of glyphosate (Wiersma 2012). Hence, fairly similar ED_{90} value for the GS population and ED_{10} values for the GR populations might suggest that kochia populations were also developing creeping resistance (gradually accruing increasing levels of resistance) to glyphosate. Considering the prevalence of the factors conducive for creeping resistance (Gressel 2009), its occurrence in western Kansas should not be surprising. Historically, growers in southwest Kansas often applied lower-than-recommended rates of glyphosate and plants were often stressed due to prevailing hot and dry conditions. Waite et al. (2013) reported no differences in absorption or translocation among kochia populations that showed differential response to glyphosate. Many other known and unknown mechanisms of glyphosate resistance are possible (Powles and Yu 2010, Perez-Jones and Mallory-Smith 2010), and cases of presence of multiple mechanisms have been reported in a few biotypes of weed species (Cocker et al.

1999, Tardif and Powles 1994). Alternatively, a few individuals in the resistant populations may have too few EPSPS gene copies to confer resistance. However, three or more EPSPS gene copies relative to ALS gene were consistently found in resistant kochia populations (Wiersma 2012).

Plants exhibited 50 to 90% more tolerance to glyphosate in an outdoor environment compared to greenhouse environment in dose-response studies. Similar results were observed in testing the 2007 collection, where many progeny plants survived up to 6.72 kg ae ha^{-1} glyphosate under outdoor environment and none of the greenhouse-grown plants survived more than 1.68 kg ae ha^{-1} glyphosate. Consequently, ED_{50} values for outdoor-grown plants were much greater than those for greenhouse-grown plants. This was expected as plants in the outdoor environment experienced temperature and moisture stress similar to field conditions. These results may help estimate the field level resistance of a population or an individual plant. Individual plants of the Scott100 population survived 6.72 kg ae ha^{-1} (ED_{90} = 7.85 kg ae ha^{-1}) glyphosate under outdoor conditions, which is 8-times greater than the normal field use rate of glyphosate. This population was among the most resistant populations based on ED_{50} or GR_{50} or GR_{90} parameters of the dose-response analysis. In contrast, ED_{90} values (under greenhouse condition) for some other resistant populations were much less (Lane101 = 0.77 and Hodgeman = 0.82 kg ae ha^{-1}) compared to that for Scott100 (3.10 kg ae ha^{-1}). This might suggest that, in addition to increased frequency of resistant individuals in the population, individual resistance level gradually increased over time under continued selection.

With several factors such as abundance of kochia, its genetic variability, and crop and weed management practices being used, the increasing occurrence of GR kochia in other parts of western Kansas and other Great Plains states is not surprising. As of 2012, presence of GR kochia populations in five other Great Plains states including Colorado, Montana, Nebraska, North Dakota and South Dakota, and Alberta Canada has been reported to the International Survey of Herbicide Resistant Weeds database (Heap 2013). While the exact mechanism is not known, considering the geographic locations of confirmed GR kochia populations, it is highly likely that the resistance developed concurrently in multiple populations. Moreover, owing to its protogynous flowers and seed dispersal mechanism, rapid local spread of the resistant biotypes is not surprising. The tumbling nature of this species may discourage growers from using proactive herbicide resistance avoidance strategies. In addition to individual growers use of best management practices, effective GR kochia management strategies should comprise collective efforts for area-wide management including roadsides and waste areas.

References

Anderson, R. L. 2004. Impact of subsurface tillage on weed dynamics in the central Great Plains. Weed Technol. 18:186-192.

Anderson, R. 1994. Characterizing weed community seedling emergence for a semiarid site in Colorado. Weed Technol. 8:245-249.

Anderson, R. and D. Nielsen. 1996. Emergence pattern of five weeds in the central Great Plains. Weed Technol. 10:744-749.

Cocker, K. M., S. R. Moss, and J. O. D. Coleman. 1999. Multiple mechanisms of resistance to fenoxaprop-p-ethyl in United Kingdom and other European populations of herbicide-resistant *Alopecurus myosuroides* (black-grass). Pestic. Biochem. Physiol. 65:3 169-180.

Crutchfield, D. A., G. A. Wicks, and O. C. Burnside. 1986. Effect of winter wheat (*Triticum aestivum*) straw mulch level on weed control. Weed Sci. 34:110-114.

Culpepper, A. S., T. L. Grey, W. K. Vencill, J. M. Kichler, T. M. Webster, S. M. Brown, A. C. York, J. W. Davis, and W. M. Hanna. 2006. Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) confirmed in Georgia. Weed Sci. 54:620-626.

Dahl, G. K., A. G. Dexter, and J. D. Nalewaja. 1982. Kochia competition and control in wheat. Proc. North Centr. Weed Contr. Conf. 37:15-16.

Dexter, A. G., J. D. Nalewaja, D. D. Rasmusson, and J. Buchli. 1981. Survey of wild oats and other weeds in North Dakota 1978 and 1979. N. D. Agric. Exp. Stn. Res. Rep. 79:1-8.

Dille, J. A., P. W. Stahlman, P. W. Geier, J. Riffel, R. S. Currie, R. G. Wilson, G. M. Sbatella, P. Westra, A. R. Kniss, M. J. Moechnig, and R. M. Cole. 2012. Kochia emergence profiles across the Central Great Plains. 2012 Weed Sci. Soc. Am. Abstr. 122. Weed Science Society of America, Lawrence, KS.

Duke, S. O. and S. B. Powles. 2008. Glyphosate: a once-in-a-century herbicide. Pest Manag. Sci. 64:319-325.

Durgan, B. R., A. G. Dexter, and S. D. Miller. 1990. Kochia (*Kochia scoparia*) interference in sunflower (*Helianthus annuus*). Weed Technol. 4:52-56.

Durham, R. M., and J. W. Durham. 1979. Kochia: its potential for forage production. Pages 444-450 *In*: Goodin, J. R. and D. K. Northington (ed.), Arid Land Plant Resources. Texas Tech University, Lubbock.

Eberle, W. M. and J. P. Shroyer. 1997. Identifying cropping-system decision factors in the wheat-fallow area of western Kansas. J. Nat. Resour. Life Sci. Educ. 26:24-28.

Eberlein, C. and Z. Fore. 1984. Kochia biology. Weeds Today. 15:5-6.

Everitt, J., M. Alaniz, and J. Lee. 1983. Seed germination characteristics of *Kochia scoparia*. J. Range Manag. 36:646-648.

Evetts, L. L. and O. C. Burnside. 1972. Germination and seedling development of common milkweed and other species. Weed Sci. 20:371-378.

Fay, P. K., D. M. Mulugeta, and W. E. Dyer. 1992. The role of seed dispersal in the spread of sulfonylurea-resistant *Kochia scoparia*. Weed Sci. Soc. Am. Abstr. 32:49.

Foes, M. J., L. Liu, G. Vigue, E. W. Stoller, L. M. Wax, and P. J. Tranel. 1999. A kochia (*Kochia scoparia*) biotype resistant to triazine and ALS-inhibiting herbicides. Weed Sci. 47:21-27.

Forcella, F. 1985. Final distribution is related to rate of spread in alien weeds. Weed Res. 25:181-191.

Friesen, L. F., H. J. Beckie, S. I. Warwick, and R. C. Van Acker. 2009. The Biology of Canadian Weeds. 138. *Kochia scoparia* (L.) Schrad. Canadian J. Plant Sci. 89:141-167.

Gaines, T. A., W. Zhang, D. Wang, B. Bukun, S. T. Chisholm, D. L. Shaner, S. J. Nissen, W. L. Patzoldt, P. J. Tranel, A. S. Culpepper, T. L. Grey, T. M. Webster, W. K. Vencill, R. D. Sammons, J. Jiang, C. Preston, J. E. Leach, and P. Westra. 2010. Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. PNAS. 107:1029-1034.

Gressel, J. 2009. Evolving understanding of the evolution of herbicide resistance. Pest Manag. Sci. 65(11):64-73.

Han, W., Z. Yang, L. Di, and R. Mueller. 2012. CropScape: A Web service based application for exploring and disseminating US conterminous geospatial cropland data products for decision support. Computers and Electronics in Agriculture. 84:111-123.

Heap, I. The International Survey of Herbicide Resistant Weeds. Online. Internet. Accessed April 17, 2013. Available www.weedscience.com

Jasieniuk, M. and B. D. Maxwell. 1994. Population genetics and the evolution of herbicide resistance in weeds. Phytoprotection. 75(4):25-35.

Keim, B. D. 2010. The lasting scientific impact of the Thornthwaite water-balance model. The Geogr. Review. 3:295-300.

Knezevic, S. Z., J. C. Streibig, and C. Ritz. 2007. Utilizing R software package for dose-response studies: The concept and data analysis. Weed Technol. 21:840-848.

Kocacinar, F. and R. Sage. 2003. Photosynthetic pathway alters xylem structure and hydraulic function in herbaceous plants. Plant Cell Environ. 26:2015-2026.

Leeson, J., A. Thomas, L. Hall, C. Brenzil, K. Brown, and R. Van Acker. 2005. Prairie weed survey: cereal, oilseed and pulse crops 1970s to the 2000s. Weed Survey Series. Agriculture and Agri-Food Canada. Saskatoon, Saskatchewan, Canada: Saskatoon Research Centre Publication 05-1. 395p.

Mengistu, L. W. and C. G. Messersmith. 2002. Genetic diversity of kochia. Weed Sci. 50:4 498-503.

Mickelson, J., A. Bussan, E. Davis, A. Hulting, and W. Dyer. 2004. Postharvest kochia (*Kochia scoparia*) management with herbicides in small grains. Weed Technol. 18:426-431.

Mulugeta, D. 1991. Management, inheritance, and gene flow of resistance to chlorsulfuron in *Kochia scoparia* L. (Schrad). M.S. thesis. Bozeman, MT: Montana State University. 134p.

Norsworthy, J. K., R. C. Scott, K. L. Smith, and L. R. Oliver. 2008. Response of north-eastern Arkansas Palmer amaranth (*Amaranthus palmeri*) accessions to glyphosate. Weed Technol. 22:408-413.

Perez-Jones, A. and C. Mallory-Smith. 2010. Biochemical mechanisms and molecular basis of evolved glyphosate resistance in weeds species. *In*: Glyphosate Resistance in Crops and Weeds: History, Development, and Management. Ed Nandula, V. K. John Wiley & Sons, Inc. pp 199-148.

Perez-Jones, A., A. W. Park, J. Colquhoun, C. Mallory-Smith, and D. L. Shaner. 2005. Identification of glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in Oregon. Weed Sci. 53:775-779.

Peterson, D. E. 1999. The impact of herbicide-resistant weeds on Kansas agriculture. Weed Technol. 13:632-635.

Powles, S. B. and Q. Yu. 2010. Evolution in action: Plants resistant to herbicides. Annu. Rev. Plant Biol. 61:317-47.

R Development Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2010.

Ritz, C. and J. C. Streibig. 2005. Bioassay analysis using R. J. Stat. Softw. 12:1-22.

Schwinghamer, T. D. and R. C. Van Acker. 2008. Emergence timing and persistence of kochia (*Kochia scoparia*). Weed Sci. 56:37-41.

Seefeldt, S. S., J. E. Jensen, and P. Fuerst. 1995. Log-logistic analysis of herbicide dose-response relationship. Weed Technol. 9:218-227.

Shaner, D. L., T. Nadler-Hasser, and C. H. Koger. 2005. A rapid in vivo shikimate accumulation assay with excised leaf discs. Weed Sci. 53:769-774.

Stallings, G. P., D. C. Thill, C. A. Mallory-Smith, and B. Shafii. 1995. Pollen-mediated gene flow of sulfonylurea-resistant kochia (*Kochia scoparia*). Weed Sci. 43:95-102.

Tardif, F. J. and S. B. Powles. 1994. Herbicide multiple-resistance in a *Lolium rigidum* biotype is endowed by multiple mechanisms: isolation of a subset with resistant acetyl-CoA carboxylase. Physiologia Plantarum. 91:488-494.

Thompson, C., D. Thill, and B. Shafi. 1994. Germination characteristics of sulfonylurea-resistant and -susceptible kochia (*Kochia scoparia*). Weed Sci. 42:50-56.

Urbano, J. M., A. Borrego, V. Torres, J. M. Leon, C. Jimenez, G. Dinelli, and J. Barnes. 2007. Glyphosate-resistant hairy fleabane (*Conyza bonariensis*) in Spain. Weed Technol. 21:396-401.

VanGessel, M. J. 2001. Glyphosate-resistant horseweed from Delaware. Weed Sci. 49:703-705.

Waite, J., C. R. Thompson, D. E. Peterson, R. S. Currie, B. L. S. Olson, P. W. Stahlman, and K. Al-Khatib. 2013. Differential kochia (*Kochia scoparia*) populations response to glyphosate. Weed Sci. 61:193-200.

Wicks, G. A., A. R. Martin, A. E. Haack, and G. W. Mahnken. 1994. Control of triazine-resistant kochia (*Kochia scoparia*) in sorghum (*Sorghum bicolor*). Weed Technol. 8:748-753.

Wicks, G. A., R. N. Klein, and C. R. Fenster. 1984. Survey of western Nebraska wheat farmers on herbicide usage. Proc. North Centr. Weed Contr. Conf. 39:140-141.

Wiersma, A. 2012. Regional whole plant and molecular response of *Kochia scoparia* to glyphosate. M.S. thesis. Colorado State University. Fort Collins, CO. 63 p.

Table 1.1 Response of 265 kochia progeny from parental plants that survived three in-season glyphosate applications in GR soybean in northwest Kansas.

Date applied	Glyphosate rate	Adjuvant	Survival count
	kg ae ha^{-1}	w/v	
31 May, 2008	0.56	-	256*
12 June, 2008	0.84	2% AMS	223
28 June, 2008	1.12	2% AMS	223

*7 unaffected, 249 injured but recovered

Abbreviations: AMS, ammonium sulfate

Table 1.2 Parameter estimates of dose-response curves for kochia mortality based on a three-parameter log logistic model (Equation 1.1) and resistance index (Equation 1.2). Values in parenthesis are ±1 standard error.

	Parameter estimates					
Population	*b*	*d*	ED_{10}	ED_{50}	ED_{90}	RI
			-------------------- kg ae ha^{-1} --------------------			
Ellis	-11	100	0.14 (0.17)	0.17 (0.00)	0.21 (0.27)	1
Hodgeman	-19	94	0.65 (0.12)	0.73 (0.31)	0.82 (0.85)	4.3 ***
Lane101	-15	94	0.58 (0.40)	0.67 (0.03)	0.77 (0.48)	4.0 ***
Lane102	-4	95	0.77 (0.12)	1.35 (0.12)	2.37 (0.65)	8.0 ***
Norton	-3	101	0.29 (0.04)	0.54 (0.04)	1.03 (0.13)	3.3 ***
Phillips	-14	100	0.64 (0)	0.75 (0)	0.89 (0)	4.5 ***
Russell	-3	95	0.53(0.08)	1.06 (0.09)	2.12 (0.46)	6.3 ***
Scott100	-2	101	0.39 (0.08)	1.11 (0.15)	3.10 (1.13)	6.6 ***
Scott99	-3	102	0.58 (0.08)	1.10 (0.08)	2.09 (0.32)	6.6 ***

Abbreviations: *b*, slope of the curve; *d*, upper response limit; ED_{10}, glyphosate dose required to cause 10% mortality; ED_{50}, glyphosate dose required to cause 50% mortality; ED_{90}, glyphosate dose required to cause 90% mortality.

***, RI significantly greater than 1 at $p<0.001$

Table 1.3 Parameter estimates of dose-response curves for kochia aboveground dry biomass based on a three-parameter log logistic model (Equation 1.1) and resistance index (Equation 1.2). Values in parenthesis are ±1 standard error.

	Parameters estimates					
Population	*b*	*d*	GR_{10}	GR_{50}	GR_{90}	RI
			-------------------- kg ae ha^{-1}	--------------------		
Ellis	2.3	100	0.03 (0.01)	0.09 (0.01)	0.23 (0.06)	1
Hodgeman	3.6	98	0.23 (0.05)	0.43 (0.05)	0.79 (0.16)	4.8 ***
Lane101	2.0	99	0.16 (0.06)	0.47 (0.08)	1.40 (0.37)	5.4 ***
Lane102	1.7	107	0.24 (0.08)	0.84 (0.14)	3.03 (0.77)	9.5 ***
Norton	2.6	98	0.21 (0.07)	0.49 (0.08)	1.16 (0.25)	5.5 ***
Phillips	4.3	100	0.35 (0.10)	0.59 (0.06)	0.99 (0.19)	6.7 ***
Russell	2.1	108	0.15 (0.04)	0.41 (0.05)	1.19 (0.36)	4.7 ***
Scott100	3.3	102	0.42 (0.14)	0.83 (0.10)	1.63 (0.36)	9.4 ***
Scott99	5.5	100	0.64 (0.11)	0.95 (0.10)	1.42 (0.21)	10.8 ***

Abbreviations: *b*, slope of the curve; *d*, upper response limit; GR_{10}, glyphosate dose required to cause 10% growth reduction; GR_{50}, glyphosate dose required to cause 50% growth reduction; GR_{90}, glyphosate dose required to cause 90% growth reduction.

***, RI significantly greater than 1 at $p<0.001$

Table 1.4 Parameter estimates of dose-response curves for kochia mortality based on a three-parameter log logistic model (Equation 1.1) and resistance index (Equation 1.2). Values in parenthesis are ±1 standard error.

GROWING CONDITION	Parameters estimates					
Population	*b*	*d*	ED_{10}	ED_{50}	ED_{90}	RI
			kg ae ha^{-1}			
GREENHOUSE						
Ellis	-4.8	101	0.29 (0.06)	0.45 (0.03)	0.71 (0.18)	1
Phillips	-3.4	102	0.98 (0.17)	1.85 (0.18)	3.51 (0.92)	4.12 ***
Scott100	-3.5	103	1.21 (0.20)	2.27 (0.23)	4.24 (1.04)	5.05 ***
OUTDOOR						
Ellis	-11.7	100	0.69 (1.98)	0.84 (0.02)	1.01 (0.89)	1
Phillips	-3.4	102	1.46 (0.24)	2.77 (0.24)	5.24 (0.96)	3.29 ***
Scott100	-3.5	101	2.24 (0.32)	4.19 (0.48)	7.85 (2.06)	4.99 ***

Abbreviations: *b*, slope of the curve; *d*, upper response limit; ED_{10}, glyphosate dose required to cause 10% mortality; ED_{50}, glyphosate dose required to cause 50% mortality; ED_{90}, glyphosate dose required to cause 90% mortality.

***, RI significantly greater than 1 at $p<0.001$

Table 1.5 Parameter estimates of dose-response curves for kochia aboveground dry wt. biomass based on three-parameter log logistic model (Equation 1.1) and resistance index (Equation 1.2) in whole plant dose-response study under greenhouse and outdoor conditions. Values in parenthesis are ±1 standard error.

GROWING CONDITION Population	Parameters estimates					
	b	*d*	GR_{10}	GR_{50}	GR_{90}	RI
			---------------------- kg ae ha^{-1}	----------------------		
GREENHOUSE						
Ellis	3.4	100	0.16 (0.02)	0.30 (0.02)	0.57 (0.05)	1
Phillips	3.8	102	0.69 (0.07)	1.23 (0.06)	2.18 (0.16)	4.1 ***
Scott100	2.7	102	0.62 (0.08)	1.38 (0.08)	3.10 (0.28)	4.6 ***
OUTDOOR						
Ellis	5.3	99	0.31 (0.03)	0.47 (0.02)	0.72 (0.09)	1
Phillips	4.0	101	1.14 (0.10)	1.98 (0.08)	3.34 (0.32)	4.1 ***
Scott100	3.4	100	1.34 (0.15)	2.56 (0.13)	4.86 (0.43)	5.4 ***

Abbreviations: *b*, slope of the curve; *d*, upper response limit; GR_{10}, glyphosate dose required to cause 10% growth reduction; ED_{50}, glyphosate dose required to cause 50% mortality GR_{50}, glyphosate dose required to cause 50% growth reduction; GR_{90}, glyphosate dose required to cause 90% growth reduction.

***, RI significantly greater than 1 at $p<0.001$

Table 1.6 Survival of selfed kochia progeny of Ellis, Lane101, and Phillips populations treated with 0.84 kg ae ha^{-1} glyphosate at 2 WAT. Ellis is a known glyphosate-susceptible population, and Phillips and Scott100 are suspected glyphosate-resistant populations collected in fall 2010 in western Kansas.

Population	Plant ID	Total number	% Survival
Ellis	1	16	0
	2	29	0
Lane101	1	21	81
	2	13	85
	3	18	89
	4	31	90
Phillips	1	22	100
	2	17	100

Abbreviations: GS, glyphosate-susceptible; GR glyphosate-resistant; WAT, weeks after treatment

Figure 1.1 Trail of kochia plants in a glyphosate-resistant (GR) soybean field near Colby, KS in 2007 that survived three in-season glyphosate applications. The dispersal plant came from an adjacent GR corn field**.**

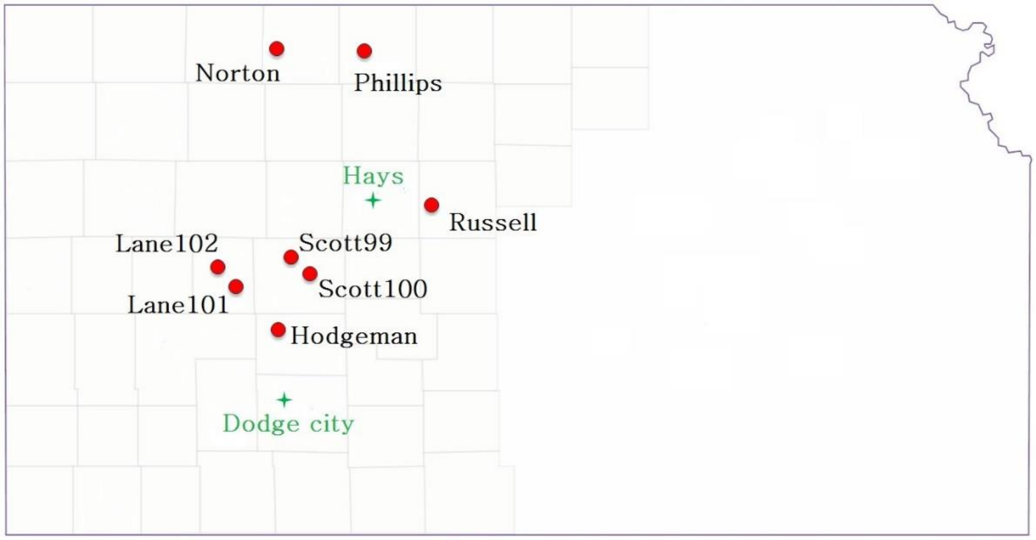


Figure 1.2 Sites in western Kansas of suspected glyphosate-resistant kochia seed collection in fall 2010.

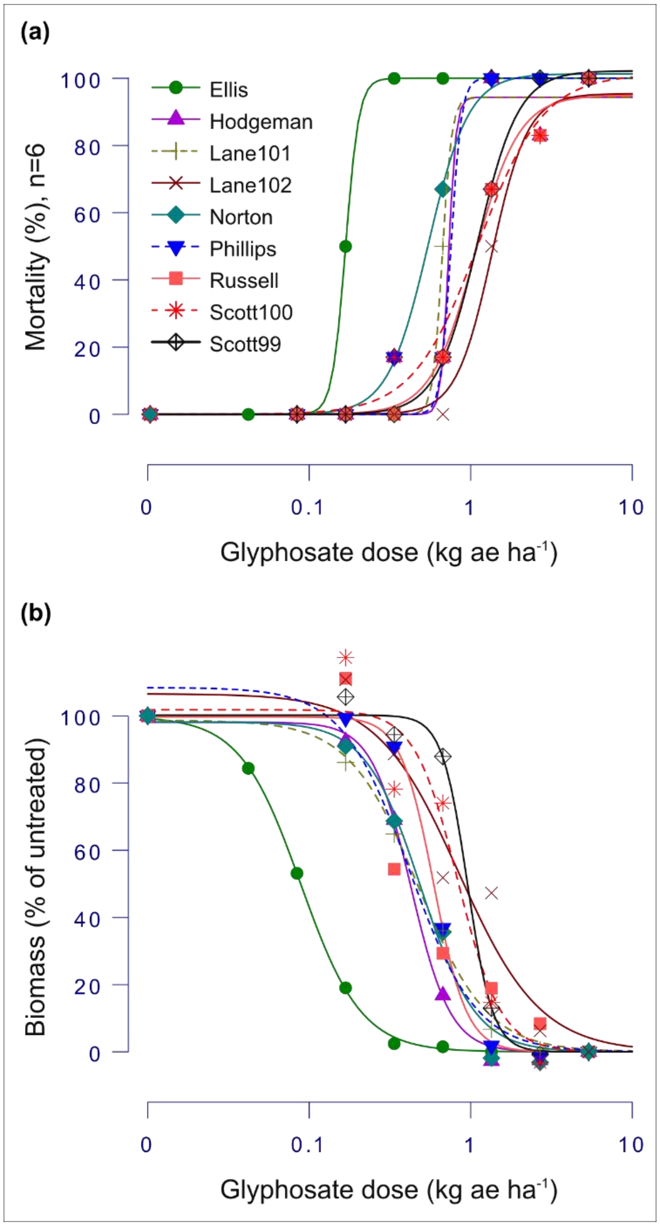


Figure 1.3 Dose-response of one glyphosate-susceptible (Ellis) and eight suspected glyphosate-resistant kochia populations collected in fall 2010 from western Kansas to glyphosate. The lines represent response curves and were generated using a three-parameter log logistic regression model (Equation 1.1). Symbols represent (a) percent mortality of populations (n=6) and (b) aboveground dry biomass (% of untreated) (n=6).

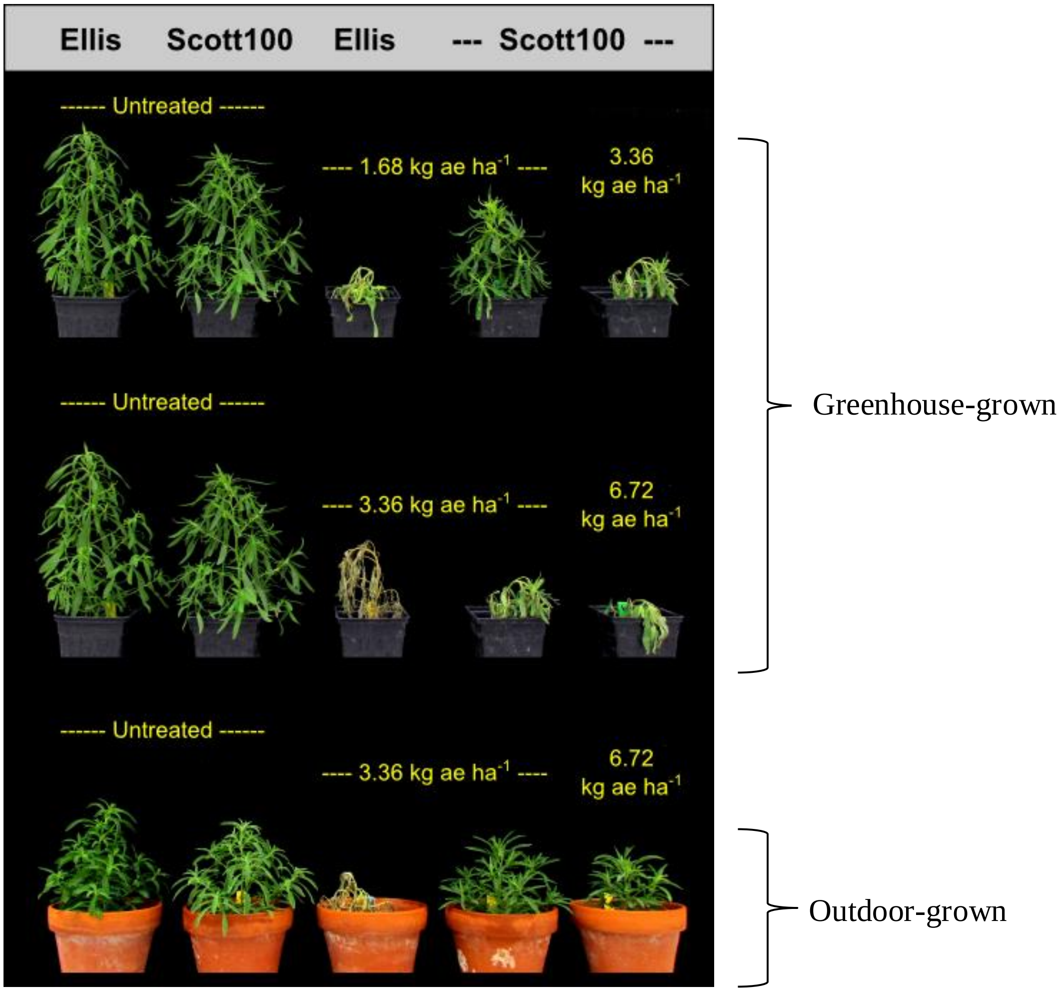


Figure 1.4 Photographs of glyphosate-susceptible population (Ellis) and one suspected glyphosate-resistant kochia population (Scott100) collected in fall 2010 from western Kansas 8 d after treatment with glyphosate.

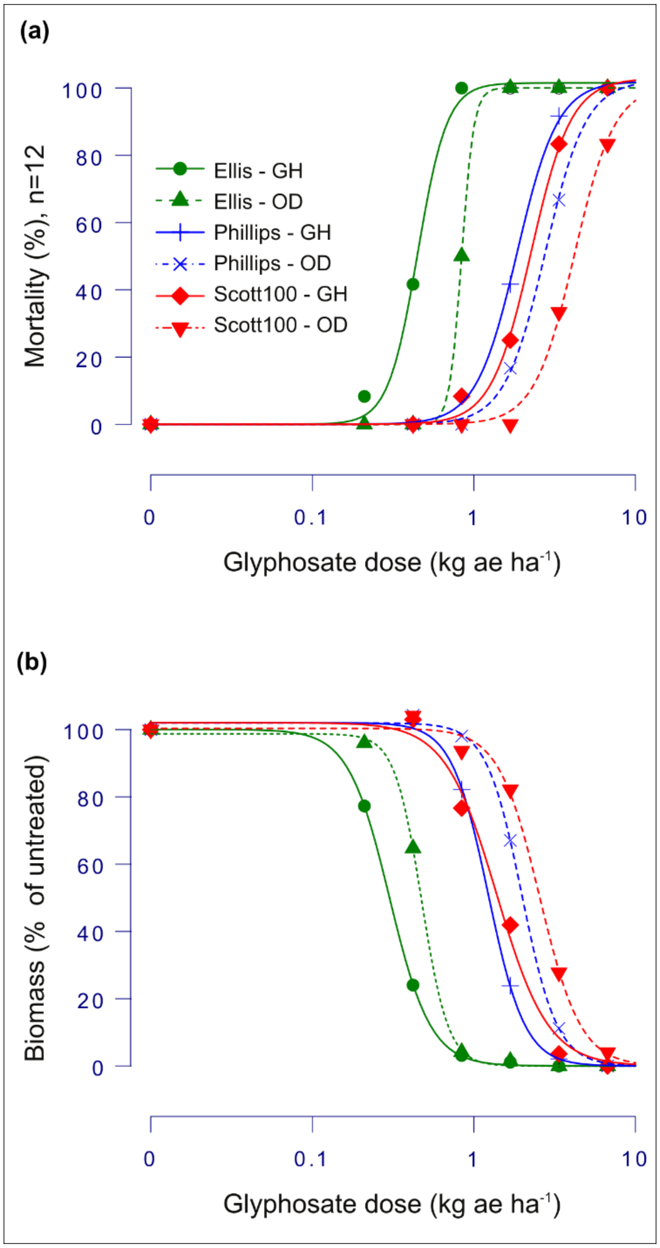


Figure 1.5 Dose-response of one glyphosate-susceptible (Ellis) and two suspected glyphosate-resistant kochia populations collected in fall 2010 from western Kansas to glyphosate under greenhouse (GH) and outdoor (OD) environments. The lines represent response curves and were generated using a three-parameter log logistic regression model (Equation 1.1). Symbols represent (a) percent mortality of populations (n=12) and (b) aboveground dry biomass (% of untreated) (n=12).

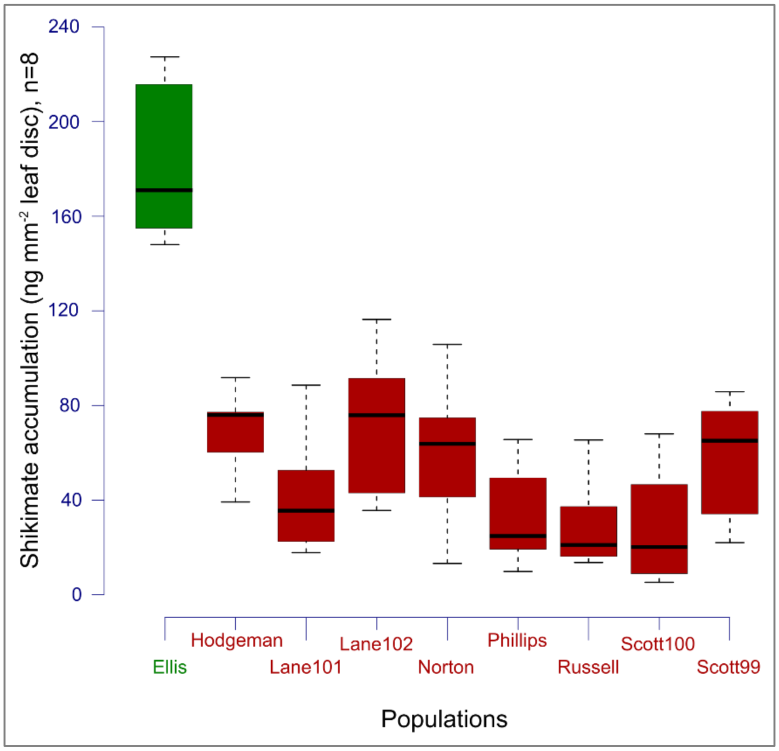


Figure 1.6 Shikimate accumulation (the amount accumulated in untreated control leaf discs subtracted) in one glyphosate-susceptible (Ellis) and eight suspected glyphosate-resistant kochia populations collected in fall 2010 from western Kansas. The 4-mm leaf discs were treated with 100 µL of 100 µM glyphosate solution under continuous light for 16 h.

The box plots show the upper (Q3) and lower (Q1) quartiles and the median. The median is identified by a line inside the box. The length of the box represents the interquartile range (middle 50% of values). The vertical lines are the full range of values in the data except outliers.

Chapter 2 - Weed Management as Affected by Increasing Presence of Glyphosate-resistant Kochia in Kansas

Abstract

Reports of incomplete to poor control of kochia [*Kochia scoparia* (L.) Schrad.] with glyphosate increased dramatically in the years following confirmed presence of glyphosate-resistant (GR) populations in 2007. A visual field survey and an online survey were conducted in 2011 and 2012, respectively, to determine the level of kochia infestation and evaluate kochia control effectiveness in wheat stubble fields, gather information on grower weed management practices during pre- and post-GR kochia confirmation periods, and evaluate the efficacy of kochia management practices during 2011-2012. Results of the visual field survey showed pervasiveness of kochia in western Kansas, kochia control failures in about one-third of wheat stubble fields, and higher-than-expected use of tillage. Results of the online survey indicated increasing infestation of kochia in glyphosate-resistant (GR) crops and fallow fields from before 2007 through 2012. Growers increased glyphosate use rates and application frequencies during the survey periods (before 2007 to 2012). The results indicated that at least one-third of the cropland in western Kansas was infested with GR kochia during 2011-2012. The spread of GR kochia has resulted in changing management practices. During the survey periods, growers drastically reduced the exclusive use of glyphosate and diversified weed management practices. Though several survey respondents reported success using other herbicides in addition or in place of glyphosate in early spring, often prior to kochia emergence, many respondents reported inconsistent results with alternative kochia control practices other than tillage.

Introduction

A suspected glyphosate-resistant (GR) kochia population in a GR soybean field in Thomas County KS in 2007 was confirmed to be resistant to glyphosate (Chapter 1). Several other widely dispersed populations in western Kansas also were confirmed resistant to glyphosate in 2010, indicating widespread presence of GR kochia in Kansas (Chapter 1). Kochia is a troublesome and economically important weed in row crops, small grains, and fallow fields in western Kansas and semiarid regions of the Great Plains and western North America (Dexter et al. 1981, Friesen et al. 2008, Wicks et al. 1984). Tillage systems, climate, and ecological characteristics are major factors influencing kochia interference with crops in Great Plains agriculture. No-till favors kochia emergence (Anderson and Nielsen 1996). Most kochia emerges in early spring, however, emergence extends to late summer (Dille et al. 2012). In addition, kochia is highly adaptive in the semiarid regions of the Great Plains. Kochia also has several characteristics that favor evolution and subsequent spread of herbicide resistance. Some of these characteristics include genetic diversity within the species (Guttieri et al. 1998, Mengistu and Messersmith 2002), reproduction and dispersal characteristics (Eberlein and Fore 1984), and short seed life (Burnside et al. 1981).

Kochia is more problematic in western than eastern Kansas because of the semiarid climate and cropping systems in the western half of the state (Peterson et al. 2010). Until the late 1990's, cropping systems in western Kansas were winter wheat dominated (Eberle and Shroyer 1997). Winter wheat continues to be the major component of cropping systems in the region but in recent years growers in western Kansas have integrated GR crops, mostly corn, into the rotation. Approximately one-third of fields are no-till (Chapter 1). A fallow year is usually included in a three-year crop rotation. Thus, growers in Kansas rely heavily on glyphosate to control weeds in

GR crops (both preplant and in-crop), post-harvest wheat fields, and during summer fallow. Because of the presence of kochia populations resistant to triazine and acetolactate synthase (ALS)-inhibiting herbicides (Foes et al. 1999, Heap 2013), declining cost of glyphosate and increasing use of practices like no-till and chemical fallow, glyphosate has been the primary herbicide used for controlling weeds in many areas (Peterson et al. 2007). In addition to economic considerations, perceived simplicity determines the specific herbicides a grower will include in a weed control program (Gianessi 2005). Growers in western Kansas were successfully controlling weeds, including kochia, with recommended and less-than-recommended rates of glyphosate until the early 2000's. Besides the confirmation of several GR kochia populations (Chapter 1), 34% of the participants, mostly from western Kansas, were concerned about glyphosate-resistant kochia in a survey conducted in 2010 (Peterson et al. 2010).

In recent years, several local and regional surveys have been conducted to understand grower awareness, perceptions, and management response to the dramatic increase of GR weeds (Givens et al. 2009, 2011, Hurley et al. 2009, Prince et al. 2012a, 2012b, Shaw et al. 2009, Weirich et al. 2011, Wilson et al. 2011). In this chapter, results of two visual surveys and an online survey regarding GR kochia and its management in western Kansas are presented. The visual surveys were conducted in August 2011 in more 1,500 wheat stubble fields to determine the level of kochia infestation and evaluate kochia control effectiveness in the western half of Kansas. The online survey was conducted in fall 2012 to estimate the impact of GR kochia in western Kansas, gather information on grower weed management practices during pre- and post-GR kochia evolution periods, and evaluate the efficacy of kochia management practices during 2011-2012.

Methods

2011 Visual Field Survey

Two visual surveys were conducted in August 2011 each encompassing more than 1500 wheat stubble fields in more than 40 western Kansas counties. The first survey was done the first week of August and the second survey was done the fourth week of August. Only fields along interstate and major highways or paved county roads were scored and a high majority of the paths travelled were common in both surveys. Data gathered included post-harvest weed management practice and visual estimations of kochia infestation and effectiveness of post-harvest weed management practice. Based on weed control method employed, the fields were classified as sprayed (POST herbicide(s) applied; spray track, visible injury or weed carcasses visible), tilled (some wheat stubble remaining on the surface), and non-controlled (no visible signs of POST herbicide application(s) or tillage). For level of kochia infestation, sprayed wheat stubble fields were classified as heavily-, moderately-, or lightly-infested (Table 2.1). Because weeds in wheat stubble fields after harvest are often sprayed with POST herbicides, PRE herbicide use was assumed to be insignificant. Kochia richness in a specific county was determined using the rating criteria indicated in Table 2.1. The fields were also evaluated for efficacy of POST herbicidal control and rated as good to excellent (>80% control), fair (50-80% control) or poor (<50% control) control of kochia.

2012 Online Survey

An online survey was developed using Adobe® FormsCentral (www.Acrobat.com) in consultation with extension weed scientists and select crop consultants in Kansas (Appendix A1). A brief statement describing the purpose and objectives of the survey preceded the questionnaire (not shown here). Product rate conversion tables and explanations wherever needed were provided. The questionnaire was divided into several parts similar to the example shown in Box 2.1. Most questions were subdivided into three distinct time periods: before 2007 (i.e., before confirmation of GR kochia in Kansas), 2007 to 2010 period (i.e., more than 10 widely distributed kochia populations confirmed GR), and 2011 to 2012 (i.e., widespread presence of GR kochia throughout western Kansas). Effective response rate varied slightly among the questions and 'I do not know' response was higher for the before 2007 time period compared to later time periods. This probably reflects the relative young age and experience of some respondents. Questions were close-ended with either numerical categorical or verbal rating options.

An advance invitation, link to instructions and survey, and courtesy reminder were sent to invited survey participants via email. Email addresses of field agronomists/crop consultants were obtained from crop consulting companies or professional associations (e.g., Crop-Quest, Servi-Tech, and Kansas Association of Independent Crop Consultants). The survey was administered in September 2012. Fifty-two crop consultants from 46 western Kansas counties participated in the survey (response rate >50% and valid response=100%).

Statistical Analysis and Presentation of Results

Raw data are presented for the 2011 visual field survey. For the 2012 online survey, results are interpreted primarily based on frequency distribution for most questions. To derive an estimate of mean responses, numerical categories were transformed into mid-values for analysis wherever appropriate. In such cases, mean values were compared using one-way ANOVA or paired t-test at α=0.05. In the cases of non-normal responses, a non-parametric Wilcoxon–Mann–Whitney test was performed instead (α=0.05).

Results

2011 Visual Field Survey

Kochia was present in nearly all counties surveyed in western Kansas. In most counties, kochia was present at moderate or higher densities (Figure 2.1). By the first week of August, about half of wheat stubble fields had been sprayed with POST herbicides and 31% of fields had been tilled (Table 2.2). No attempt was made to identify the herbicides applied to sprayed fields. Occasionally, there was visual evidence that some tilled fields had been sprayed previously with poor results. Neither POST herbicide nor tillage was used to manage weeds in 21% of the fields. A high proportion of the fields in which weeds had not been controlled at the time of the first survey (early August) had been sprayed with herbicide(s) by the end of August. The proportion of tilled fields remained nearly the same. Of the sprayed fields, 4% were heavily infested, 52% were moderately infested, and 44% were lightly infested with kochia (data not shown). Kochia control in sprayed fields was good to excellent in 71% of the fields (Table 2.3). However, in 29% of the sprayed fields, kochia control was fair to poor. Kochia control was good to excellent in almost all tilled fields.

2012 Online Survey

Kochia Infestation and Presence of GR Kochia

Collectively, the respondents scouted >420,000 ha of cropland, which is more than 10% of the area surveyed. Seventy-six percent of respondents reported kochia infestation in >10% of fallow fields before 2007 (Figure 2.2a). More than one-fourth of respondents reported kochia infestation in >80% of fallow fields. During 2007-2010, only 7% of respondents reported kochia infestation in ≤10% fallow fields, whereas an additional 12% of respondents reported >80% kochia-infested fallow fields compared to before 2007. More than half of the respondents reported increased percentage of kochia-infested fallow fields during 2007-2010 compared to before 2007, and most reported further increased infestation during 2011-2012 (Figure 2.2b). However, most respondents who reported kochia infestation in ≤80% fields reported increased percentage of kochia-infested fields during the survey years. Class interval means for percent kochia-infested fallow fields (>80% =90%) were 47, 57, and 70% for the before 2007, 2007-2010, and 2011-2012 time periods, respectively ($P<0.001$ for all combinations). Half of the respondents reported different levels of infestation in fallow and GR crops during 2011-2012, however, the class interval mean for infestation was similar in fallow and GR fields (Figure 2.2c).

Almost all respondents reported presence of GR kochia ranging from 1 to >50% of fields during 2011-2012 (Figure 2.3). While 88% of respondents reported GR kochia in more than 20% of the fields, half of them reported GR kochia in >50% of the fields. The class interval mean for GR kochia infested fields was 46% of all kochia infested fields.

Glyphosate Use Rate and Application Frequency

The most common recommended use rate of glyphosate is 0.84 kg ae ha^{-1}. For convenience of reporting survey results, glyphosate rates of 0.79 to 0.95 kg ae ha^{-1} were considered normal use rates. Responses for glyphosate use rate in fallow fields differed among the three time periods ($p<0.001$ for all combinations) (Figure 2.4). Eighty-six percent of respondents reported glyphosate use rate per application in fallow fields was ≤0.95 kg ae ha^{-1} before 2007. Percentage of respondents reporting similar use rates decreased to 61% and 21% during 2007-2010 and

2011-2012, respectively. Seventy-eight and 90% percent of respondents reported increased glyphosate use rate during 2007-2010 and 2011-2012, respectively, compared to that before 2007. While only 2% reported $\geq$1.36 kg ae ha^{-1} use rates of glyphosate before 2007, 34% reported those higher use rates during 2010-2012. Responses for glyphosate use rate in GR crops also differed among three time periods ($p<0.001$ for all combinations) (Figure 2.5). However, the responses did not differ between the corresponding time periods in fallow and GR crops ($P>0.1$ for all combinations). The combined class arithmetic mean values (>1.58=1.68 kg ae ha^{-1}) for glyphosate use rates in fallow and GR crops were 0.8, 0.98, and 1.22 kg ae ha^{-1} for before 2007, 2007-2010, and 2011-2012 time periods, respectively.

Glyphosate application frequency per season in fallow fields averaged 2.0, 2.6, and 2.9 for the before 2007, 2007-2010, and 2011-2012 time periods, respectively ($P<0.001$ for all combinations) (Figure 2.6). Respondents reporting three or more applications of glyphosate per season increased from 28% before 2007 to 51 and 72% during 2007-2010 and 2011-2012, respectively. While more than half of respondents reported increased application frequency of glyphosate during 2007-2010, more than two-thirds reported increased application frequency during 2011-2012 compared to before 2007. Most fields that had received three applications of glyphosate before 2007 also received glyphosate in the same frequency during later periods. Responses for glyphosate application frequencies in GR crops were not similar to that for fallow fields during corresponding time periods ($R^2<0.15$ for all) (Figure 2.7). However, average application frequencies were similar in fallow and GR fields during corresponding time periods ($P>0.5$ for all combinations).

Absolute Dependency on Glyphosate and Efficacy with and without Dicamba

Frequency distribution for absolute dependency on glyphosate use in GR crops differed among the three periods ($P<0.001$ for all combinations) (Figure 2.8). For the period before 2007, the percentage of GR fields that had received glyphosate exclusively varied widely among respondents, mostly ranging from 11 to 80% of fields, of which more than half reported exclusive use of glyphosate in >50% of the GR fields. However, during 2007-2010, three-fourths of respondents reported exclusive glyphosate use on ≤50% of the fields. Continuing this decreasing trend, only 25% of respondent reported exclusive glyphosate use on ≤20% of fields during 2011-2012. The class interval arithmetic mean for total dependency on glyphosate in GR crops (>80%=90%) decreased from 49 to 15% of the fields during the survey years.

With glyphosate-only applications, 85% percent of respondents reported effective control of kochia in most fields or throughout the county before 2007 (Figure 2.9a). The percentage of respondents reporting similar kochia control decreased to 44% during 2007-2010 and 8% during 2011-2012. In contrast, the percentage of respondents reporting unsatisfactory or ineffective kochia control increased from 12% before 2007 to 92% during 2011-2012. With glyphosate plus dicamba POST applications, >90% respondents reported effective control of kochia throughout the county or region before 2007 (Figure 2.9b). The percentage of respondents reporting similarly effective kochia control with glyphosate plus dicamba applications did not change much during 2007-2010, but decreased to only 21% during 2011-2012, with more than three-fourths of respondents reporting unsatisfactory or ineffective kochia control.

Difficulty in Controlling Kochia

Seventy-seven percent of respondents rated difficulty of controlling kochia in fallow fields as “mostly controlled” before 2007 and about one-fourth rated as “frequently controlled” (Figure

2.10a). While no one answered “occasionally” or “rarely controlled” before 2007, 34 and 78% reported ineffective control during 2007-2010 and 2011-2012, respectively. Furthermore, half reported that kochia was rarely controlled during 2011-2012. Responses were similar for GR crops, regardless of the time periods (Figure 2.10b).

Management Practices and Their Effectiveness

During 2011-2012, more than half of respondents reported normal glyphosate use rate in ≤10% of fields (Figure 2.11a). Other respondents reported higher-than-normal glyphosate use rate in >60% of fields. Three-fourths of respondents reported multiple applications of glyphosate in >60% of fields. A majority reported that mixtures of glyphosate plus dicamba (>0.25 kg ae ha^{-1} dicamba) were applied in >40% of fields. Response for other POST and PRE herbicide applications were similar to those for multiple glyphosate applications and higher rates of glyphosate, respectively. More than three-fourths reported kochia control with tillage in ≤40% of the fields. Class interval arithmetic means for kochia management practices (>60%=75%)were 27, 52, 56, 50, 57, 52, and 23% for normal rate of glyphosate, higher-than-normal rate of glyphosate, multiple applications of glyphosate, glyphosate plus dicamba, other POST herbicides, PRE herbicides, and tillage, respectively.

Regardless of use rate and application frequency, about three-fourths of respondents reported glyphosate applications were effective in only half or less of fields (Figure 2.11b). Higher-than-normal glyphosate use rates were effective in a higher percentage of fields compared to normal use rate or multiple applications of glyphosate. Respondents reported greater kochia control when tank mixing >0.25 kg ae ha^{-1} dicamba with glyphosate, however, only 50% reported such applications as being effective in most fields. More than half and about three-fourths reported effective kochia control in most fields with other POST or PRE herbicides, respectively. Respondents reported greater kochia control with PRE herbicide use compared to POST herbicide applications, yet, at least one-fourth reported those applications effective in only half or less of fields. Not surprisingly, most of respondents reported that tillage was effective in most fields.

Respondents were also asked to answer the most effective kochia management practice(s) used in 2012 not commonly used in prior years. Most respondents reported a variety of PRE and POST herbicides. In general, respondents reported effective kochia control with PRE followed by POST herbicide applications. Many emphasized early herbicide applications staring in February to early April. In general, they reported either commercial products with multiple ingredients for both PRE and POST herbicides, or tank mixing of two or more different herbicides.

Discussion

The 2011 visual field survey indicated that kochia is prevalent throughout western Kansas. A majority of wheat stubble fields were infested with moderate to high densities of kochia. The distribution was not uniform among counties; however, kochia was present in almost all counties with at least half of the counties having moderate to high density infestations. A large majority of wheat stubble fields (64%) had been sprayed with unknown herbicides for post-harvest weed control; yet, more fields than expected (34%) had been tilled by the last week of August. The higher-than-expected use of tillage may indicate a shift towards increased use of tillage to control kochia. In western Kansas, weeds are commonly managed chemically both in crop and fallow fields (chemical fallow). In about one-fourth of the total sprayed fields, POST herbicides had been applied in August. This could be due to three possible reasons: 1) earlier applications were not effective, 2) to avoid adverse mid-summer environmental conditions, and 3) intentional delay to avoid omission of later emerging weeds. Unsatisfactory kochia control in nearly one-third of the fields and extended emerging patterns of kochia supports these hypotheses.

The 2012 online survey also indicated the prevalence of kochia throughout western Kansas. Almost all respondents reported kochia presence to some extent throughout the survey periods. However, kochia infestation was not uniform throughout the region. The distribution level of kochia infestation in the region matches fairly well with results of the visual field survey (data not shown). Interestingly, most respondents, especially those who reported kochia infestation in ≤80% of the fields before 2007, reported increased infestation of kochia during the survey years. The reason for increased infestation of kochia may not be exclusively attributed to increasing presence of GR kochia as many growers did not apply herbicides in wheat in 2010 because of the poor condition of the wheat crop, thereby, allowing more kochia establishment than usual in more normal years. Nonetheless, weed occurrence in a given area increases following evolution of herbicide resistance. In this survey, almost all respondents reported presence of GR kochia in at least a few fields during the latter years, with a majority reporting GR kochia in >50% of the fields, thus, indicating widespread presence of GR kochia in western Kansas.

Glyphosate use rate increased during the survey years. In general, normal or below normal glyphosate use rates had been applied in both fallow and GR crops before 2007. Glyphosate use rate increased slightly during 2007-2010; still, a majority of respondents reported use of normal or lower-than-normal use rates of glyphosate during that period. During 2011-2012, however, glyphosate use rates were increased in almost all fields compared to use rates before 2007, with more than three-fourths applying higher-than-the normal use rates. The low rates of glyphosate applied during the earlier years might have facilitated evolution of glyphosate resistance in kochia. For proactive pest resistance management, Gressel (2011) suggested elimination of the high mutation rates in pests by avoiding low application rates of pesticides. However, increased glyphosate use rates during the latter periods appear to be a reactive rather than proactive strategy. Dose response studies indicated that twice the normal rate of glyphosate was lethal to a significant proportion of individuals in many 2010 GR kochia populations from western Kansas (Chapter 1). However, partial and ineffective kochia control was observed in several fields in the 2011 visual survey, though the herbicides applied were not known. Continued selection pressure with higher rates of the same herbicide thrusts populations towards higher levels of resistance. Thus, the gain, if any, with increased rate of glyphosate would be no more than ephemeral. Correspondingly, both individual and population level differences were observed in GR kochia populations (Chapter 1), which may indicate discrepancy in selection pressures on those populations.

Together with use rate, glyphosate application frequency also increased during the survey years. While one or two applications of glyphosate were common before 2007, a majority of growers applied three or four applications of glyphosate during 2011-2012. Kochia plants that survived the first application of glyphosate also survived second and third applications of even higher rates (Chapter 1). However, such practices might partially control newly emerged kochia plants. In the same way, respondents reported better kochia control with higher rates of glyphosates than with the multiple applications.

Results of the online survey indicated that glyphosate was exclusively used for controlling weeds in nearly half of GR crop fields prior to 2007. Similarly, in a six-state grower survey, Wilson et al. (2011) reported higher reliance on glyphosate for weed management in GR crops. Hurley et al. (2009), Johnson et al. (2007), Scott and VanGessel (2007), and Young (2006) also reported total dependency on glyphosate in a majority of GR crops. Sole dependency or exclusive use of glyphosate decreased considerably by the end of the online survey period. This indicates that growers were not only increasing the use rate and frequency of glyphosate applications, but they also were using alternatives during the later years. The percentage of respondents reporting effective kochia control with glyphosate-only application decreased from 85% to 8% during the survey years. The continued failure with glyphosate-only applications resulted in a shift towards alternative practices. Dicamba is often included in POST herbicide applications in fallow lands. Dicamba resistance was reported in a kochia population in Nebraska several years ago (Cranston et al. 2001). Not surprisingly, respondents also reported decreasing effectiveness of glyphosate plus dicamba applications during the survey years, indicating possible stacked resistance to both glyphosate and dicamba in western Kansas kochia populations.

Respondents reported increasing difficulty of controlling kochia in western Kansas. Apart from recent evolution of resistance to glyphosate and possibly to dicamba, resistance to triazine and ALS-inhibiting herbicides were confirmed in Kansas decades earlier (Heap 2013). Although, difficulty in controlling kochia in this case primarily entails glyphosate resistance, presence of resistance to other modes of action unquestionably complicates choices of alternative herbicides.

Together with glyphosate applications, other POST and PRE herbicides were applied to control kochia during 2011-2012. Similarly, in a survey regarding herbicide use patterns in GR cropping systems, Prince et al. (2012b) found a higher percentage of growers integrating non-glyphosate herbicides during 2010 compared with 2005. Whereas the survey revealed that normal or lower-than-normal rates of glyphosate were commonly used before 2007, those rates were applied on less than one-fourth of the fields toward the end of the survey period. Higher-than-normal use rates and multiple applications of glyphosate, glyphosate plus dicamba, other POST herbicides, and PRE herbicides were applied on at least half of the fields in 2011-2012. This shows that, on an average, a combination of three different practices (herbicide-focused) was used, indicating use of diversified chemical approaches to kochia management. Although tillage was practiced less frequently compared to chemical methods, unexpectedly higher numbers of fields (nearly one-fourth) were tilled, presumably to control kochia. Other POST and PRE herbicides generally were more effective in controlling kochia compared to glyphosate applications, however, such practices by themselves were not effective on about one-third of fields. Combinations of practices, as discussed above, presumably improved control of kochia. However, greater than expected use of tillage might suggest kochia control failures with chemical methods in several cases. In contrast, no-till is considered better than conventional tillage for profitability and sustainability of Great Plains agriculture. Many survey respondents reported success with early applications of herbicides and/or use of multiple modes of actions.

Kochia was a fairly common weed in western Kansas before the evolution of GR populations. Occurrence of GR kochia populations increased rapidly in the past few years and has now become widespread in western Kansas. Kochia infestation in GR crops and fallow lands generally increased with presence of GR kochia. Growers increased both the use rate and frequency of glyphosate applications to manage kochia; however, success was limited in less than half of the fields during the end of the survey period. Other POST or PRE herbicides helped better manage kochia populations to some extent. Although not recommended for soil and water conservation reasons, tillage was done in substantial numbers of fields and was mostly effective. Many reported success with overlapping applications of PRE and POST herbicides in controlling kochia, especially with early applications and multiple modes of action. Such practices, in most instances, incur considerably higher cost compared to previous cost of using glyphosate alone. Survey result indicates that growers primary concern in implementing herbicide resistance management practices in GR cropping system is the cost involved (Givens 2010). In a survey also focusing on GR cropping systems, Weirich et al. (2011) concluded that growers should adopt weed resistance best management practices (BMP), regardless of risk behavior or profitability. Nonetheless, considering kochia's economic importance in semiarid agriculture, its history of herbicide resistance evolution, and its emergence patterns, sustainable kochia management strategies should incorporate diversified approaches. Most importantly, diversifying herbicides help reduce population densities of other weeds and also lessen the potential risk for the evolution of weed populations resistant to other modes of action. Results from this survey may be useful for extension specialists, researchers, and agro-industries for more focused information delivery and developing recommendation practices, further research, and more customized customer service.

References

Anderson, R. and D. Nielsen. 1996. Emergence pattern of five weeds in the central Great Plains. Weed Technol. 10:744-749.

Burnside, O. C., C. R. Fenster, L. L. Evetts, and R. F. Mumm. 1981. Germination of exhumed weed seed in Nebraska. Weed Sci. 29:577-586.

Cranston, H. J., A. J. Kern, J. L. Hackett, E. K. Miller, B. D. Maxwell, and W. E. Dyer. 2001. Dicamba resistance in kochia. Weed Sci. 49:164-170.

Dexter, A. G., J. D. Nalewaja, D. D. Rasmusson, and J. Buchli. 1981. Survey of wild oats and other weeds in North Dakota 1978 and 1979. N. D. Agric. Exp. Stn. Res. Rep. 79:1-8.

Dille J. A., P. W. Stahlman, P. W. Geier, J. Riffel1, R. S. Currie, R. G. Wilson, G. M. Sbatella, P. Westra, A. R. Kniss, M. J. Moechnig, and R. M. Cole. 2012. Kochia emergence profiles across the Central Great Plains. 2012 Weed Sci. Soc. Am. Abstr. 122. Weed Science Society of America, Lawrence, KS.

Eberle, W. M. and J. P. Shroyer. 1997. Identifying cropping-system decision factors in the wheat-fallow area of western Kansas. J. Nat. Resour. Life Sci. Educ. 26:24-28.

Eberlein, C. V. and Z. Q. Fore. 1984. Kochia biology. Weeds Today 15(2): 5-7.

Foes, M. J., L. Liu, G. Vigue, E. W. Stoller, L. M. Wax, and P. J. Tranel. 1999. A kochia (*Kochia scoparia*) biotype resistant to triazine and ALS-inhibiting herbicides. Weed Sci. 47:21-27.

Friesen, L. F., H. J. Beckie, S. I. Warwick, and R. C. Van Acker. 2008. The Biology of Canadian Weeds. 138. *Kochia scoparia* (L.) Schrad. Canadian J. Plant Sci. 89:141-167.

Gianessi, L. P. 2005. Economic and herbicide use impacts of glyphosate-resistant crops. Pest Manage. Sci. 61:241-245.

Givens, W.A. 2010. Shifts in herbicide use, tillage practices, and perceptions of glyphosate-resistant weeds following adoption of glyphosate-resistant crops. PhD Dissertation, Mississippi State University, Starkville, MS.

Givens, W. A., D. R. Shaw, and M. E. Newman. 2011. Benchmark study on glyphosate-resistant cropping systems in the USA. III. Grower awareness, information sources, experiences, and management practices regarding glyphosate-resistant weeds. Pest Manag. Sci. 67:758-770.

Givens, W. A., D. R. Shaw, W. G. Johnson, S. C. Weller, B. G. Young, R. G. Wilson, M. D. K. Owen, and D. Jordan. 2009. A grower survey of herbicide use patterns in glyphosate-resistant cropping systems. Weed Technol. 23:156-161.

Gressel, J. 2009. Evolving understanding of the evolution of herbicide resistance. Pest Manag. Sci. 65:64-73.

Guttieri, M. J., C. V. Eberlein, and E. J. Souza. 1998. Inbreeding coefficients of populations of *Kochia scoparia* using chlorsulfuron resistance as a phenotypic marker. Weed Sci. 46:521-525.

Heap, I. The International Survey of Herbicide Resistant Weeds. Online. Internet. Accessed Friday, May 3, 2013. Available www.weedscience.com

Hurley, T. M., P. D. Mitchell, and G. B. Frisvold. 2009. Weed management costs, weed best management practices, and the Roundup Ready® weed management program. AgBioForum 12:281-290.

Johnson, W. G., K. D. Gibson, and S. P. Cowley. 2007. Does weed size matter? An Indiana grower perspective about weed control timing. Weed Technol. 21:542-546.

Peterson, D., C. Thompson, D. Shoup, B. Olson, J. Falk, and K. Martin. 2010. Grower, crop advisor, and extension agent perceptions about glyphosate resistant weeds in Kansas. Proc. WSWS 63:105.

Peterson, D., B. Olson, K. Al-Khatib, R. Currie, J. A. Dille, J. Falk, P. Geier, D. Regehr, P. Stahlman, and C. Thompson. 2007. Glyphosate Stewardship: Optimizing and Preserving Glyphosate Performance. KCES MF-2767.

Prince, J. M., D. R. Shaw, W. A. Givens, M. D. K. Owen, S. C. Weller, B. G. Young, R. G. Wilson, and D. L. Jordan. 2012a. Benchmark Study: I. Introduction, weed population, and management trends from the benchmark survey 2010. Weed Technol. 26:525-530.

Prince, J. M., D. R. Shaw, W. A. Givens, M. E. Newman, M. D. K. Owen, S. C. Weller, B. G. Young, R. G. Wilson, and D. L. Jordan. 2012b. Benchmark Study: III. Survey on changing herbicide use patterns in glyphosate-resistant cropping systems. Weed Technol. 26:536-542.

Mengistu, L. W. and C. G. Messersmith. 2002. Genetic diversity of kochia. Weed Sci. 50:498-503.

Scott, B. A. and M. J. VanGessel. 2007. Delaware soybean grower survey on glyphosate-resistant horseweed (*Conyza canadensis*). Weed Technol. 21:270-274.

Shaw, D. R., W. A. Givens, L. A. Farno, P. D. Gerard, D. Jordan, W. G. Johnson, S. C. Weller, B. G. Young, R. G. Wilson, and M. D. K. Owen. 2009. Using a grower survey to assess the benefits and challenges of glyphosate-resistant cropping systems for weed management in U.S. corn, cotton, and soybean. Weed Technol. 23:134-149.

Weirich, J. W., D. R Shaw, K. H. Coble, M. D. K. Owen, P. M. Dixon, S. C. Weller, B. G. Young. R. G. Wilson, and D. L. Jordan. 2011. Benchmark study on glyphosate-resistant cropping systems in the United States. Part 6: Timeliness of economic decision-making in implementing weed resistance management strategies. Pest Manag. Sci. 67:785-789.

Wicks, G. A., R. N. Klein, and C. R. Fenster. 1984. Survey of western Nebraska wheat farmers on herbicide usage. Proc. North Centr. Weed Contr. Conf. 39:140-141.

Wilson, R. G., B. G. Young, J. L. Matthews, S. C. Weller, W. G. Johnson, D. L. Jordan, M. D. K. Owen, P. M. Dixon, and D. R. Shaw. 2011. Benchmark study on glyphosate-resistant cropping systems in the United States. Part 4: Weed management practices and effects on weed populations and soil seedbanks. Pest Manag. Sci. 67:771-780.

Young, B. G. 2006. Changes in herbicide use patterns and production practices resulting from glyphosate-resistant crops. Weed Technol. 20:301-307.

Table 2.1 Rating criteria for kochia infestation at field- and county-levels in western Kansas in August 2011.

Field-level kochia infestation	Approximate kochia density in 100 m^2 area
Heavily-infested	>100
Moderately-infested	20-100
Lightly-infested	<20
County-level kochia abundance	**Description**
Very common	Most fields heavily-infested
Common	Majority of the fields heavily-infested
Moderately common	Majority of the fields moderately-infested
Not so common	Most fields lightly-infested
Rare	Kochia present in only a few fields, mostly lightly-infested

Table 2.2 Method of weed management in wheat stubble fields in western Kansas at the beginning and end of August 2011.

Time	Status of wheat stubble fields		
	Sprayed	Tilled	Non-controlled
	------------ % of fields ------------		
Beginning of August (*n=1607)*	49	31	21
End of August (*n=1503)*	64	34	5

Table 2.3 Visual evaluation of the effectiveness of kochia control practices in wheat stubble fields in western Kansas at the end of August 2011.

Control method employed	Kochia control Good to excellent	 Fair	 Poor
	------------ % fields ------------		
Sprayed fields *(n=962)*	71	28	1
Tilled fields *(n=471)*	99	1	0

PART A. Kochia infestation and presence of glyphosate resistance

A1a. In 2011 and 2012, what percentage of summer fallow fields were infested with kochia?
Options -> 0-10%; 11-20%; 21-40%; 41-60%; 61-80%; >80%; I do not know

A1b. In 2011 and 2012, what percentage of row crop fields were infested with kochia?
Options -> same as A1a.

A2. In 2012, what percentage of all fields (crop and fallow) infested with kochia do you think were glyphosate-resistant populations?
Options -> 0%; 1-5%; 6-10%; 11-20%; 21-30%; 31-40%; 41-50%; >50%; I do not know

---

PART B. Glyphosate use rate and application frequency

B1. What was the most common rate of glyphosate (kg ae/ha) applied in glyphosate-resistant crops,
a. before 2007? **b.** during 2007 to 2010? **c.** during 2011-2012? (Refer to the conversion table provided)
Options -> 0.44-0.62; 0.63-0.78; 0.79-0.95; 0.96-1.12; 1.13-1.35; 1.36-1.58; >1.58 kg ae ha^{-1}; I do not know.

B2. How many times was glyphosate applied in summer fallow in a season, **a.** before 2007? **b.** during 2007 to 2010? **c.** during 2011-2012?
Options -> 1; 2; 3; 4 applications; I do not know

PART C. Absolute dependency on glyphosate and efficacy with and without dicamba

C1. What percentage of glyphosate-resistant crop fields received glyphosate as the ONLY herbicide for in-crop weed management? Note: Do not consider POST herbicides applied for volunteer crop control.
Options -> 0-10%; 11-20%; 21-30%; 31-40% 41-50%; 51-60%; 61-70%; 71-80%; >80%; I do not know

C2a. How effective were glyphosate-only products in controlling kochia in summer fallow, **a.** before 2007? **b.** during 2007 to 2010? **c.** during 2011-2012?
Options -> very effective throughout the county; very effective in most fields; not satisfactory in several fields; not effective in several fields; I do not know.

C2b. How effective were POST applications of glyphosate PLUS dicamba products in controlling kochia in summer fallow, **a.** before 2007? **b.** during 2007 to 2010? **c.** during 2011-2012?
Options -> same as C2a.

PART D. Difficulty of controlling kochia

D1a. How difficult was it to control kochia in summer fallow**, a.** before 2007? **b.** during 2007 to 2010? **c.** during 2011-2012?.
Options -> mostly controlled; frequently controlled; occasionally controlled; rarely controlled; I do not know.

D1b. How difficult was it to control kochia in glyphosate-resistant crops? **a.** before 2007? **b.** during 2007 to 2010? **c.** during 2011-2012?
Options -> same as in D1a.

D2. How often did growers' weed management program specifically target kochia control, **a.** before 2007? **b.** during 2007 to 2010? **c.** during 2011-2012?
Options -> never; sometimes; occasionally; always; I do not know.

PART E. Management practices employed to control kochia and their effectiveness

E1. What percentage of your growers implemented the following practices to control kochia in 2011 and/or 2102,
a. normal rate of glyphosate **b.** higher than normal rate of glyphosate **c.** multiple applications of glyphosate **d.** glyphosate plus dicamba **e.** other POST herbicides **f.** PRE herbicides **g.** tillage
Options -> 0-5%; 6-10%; 11-20%; 21-40%; 41-60%; >60%; I do not know

E2. How do you rate those practices based on their effectiveness in controlling kochia in 2011 and/or 2102?
a. normal rate of glyphosate **b.** higher than normal rate of glyphosate **c.** multiple applications of glyphosate **d.** glyphosate plus dicamba **e.** other POST herbicides **f.** PRE herbicides **g.** tillage
Options -> effective in all fields; effective in most fields; effective in about half of fields; effective in only few; did not work at all; I do not know

Box 2.1 Questionnare for 2012 crop consultant survey regarding impact and management of GR kochia in western Kansas. The questionare is in its condended form and does not include instructions, background information and yes/no type questions.

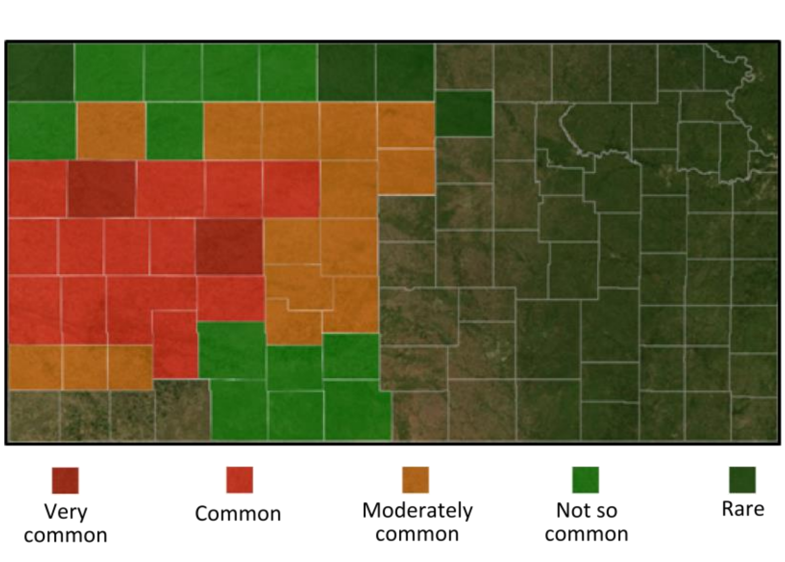


Figure 2.1 Kochia richness in western Kansas counties (refer to Table 2.1 for details).

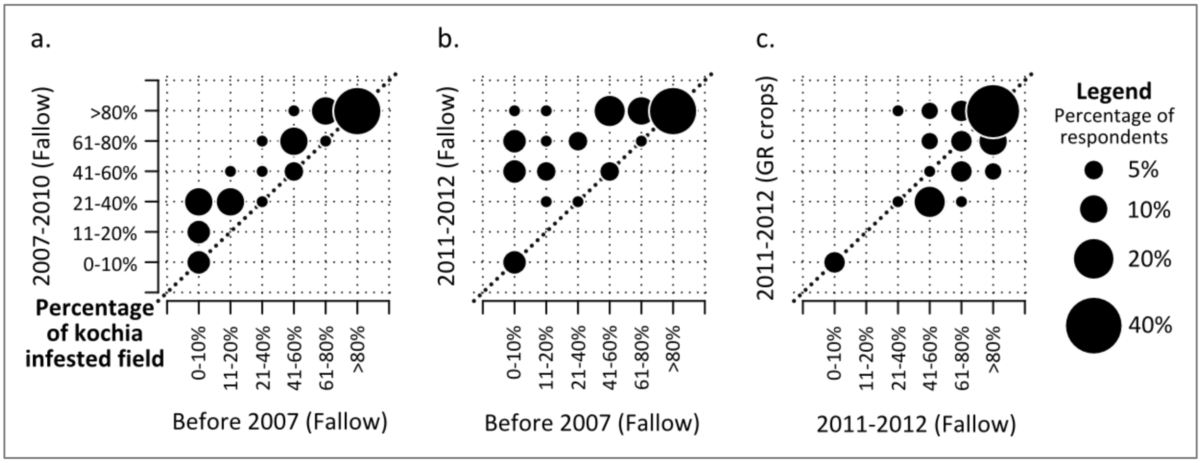


Figure 2.2 Bubble graph showing kochia infestation in fallow fields in western Kansas reported by crop consultants. Both axes are precentage of kochia-infested fields and circles represent percentage of respodents in proportion to the legend. (a) kochia infestation in fallow fields before 2007 plotted against that during 2007-2010 (n=41), (b) kochia infestation in fallow fields before 2007 plotted against that during 2011-2012 (n=41), and (c) kochia infestation during 2011-2012 in fallow fields plotted against that in GR crops (n=51).

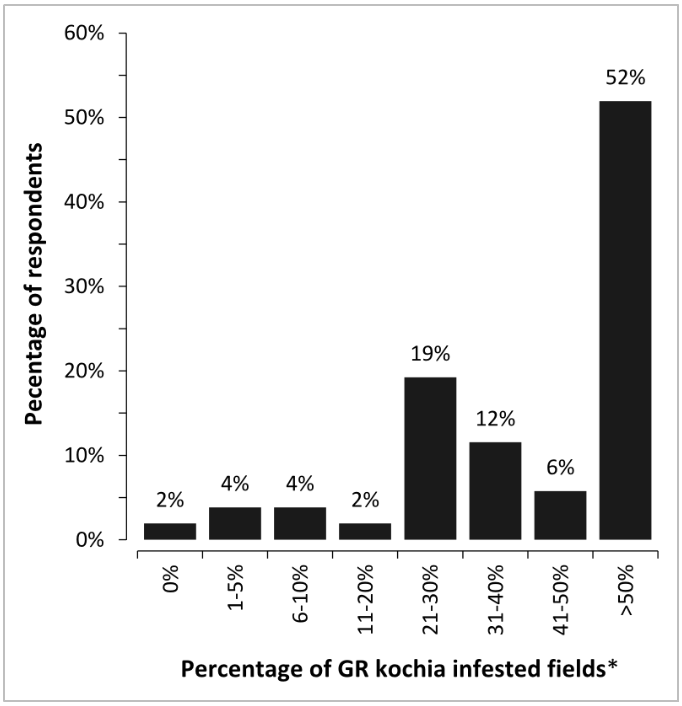


Figure 2.3 Percentage of crop consultants (n=52) reporting GR kochia-infested fields in western Kansas during 2011-2012.

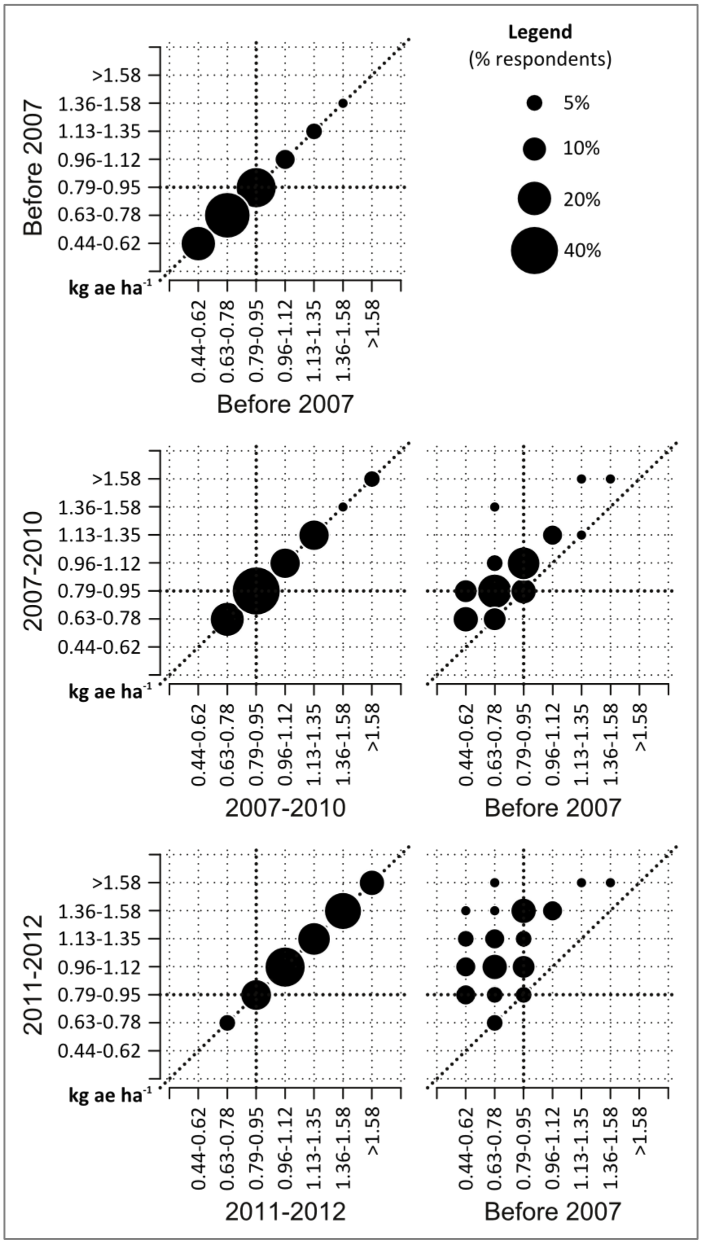


Figure 2.4 Glyphosate use rate in fallow fields in western Kansas reported by crop consultants (n=44). Both axes are glyphosate use rate in kg ae ha^{-1} and circles represent percentage of respondents in proportion to the legend. The highlighted grids represent normal use rate of glyphosate. Circles above the diagonal line indicate increased glyphosate use rate during the survey period in Y-axis compared to that during the survey period in X-axis.

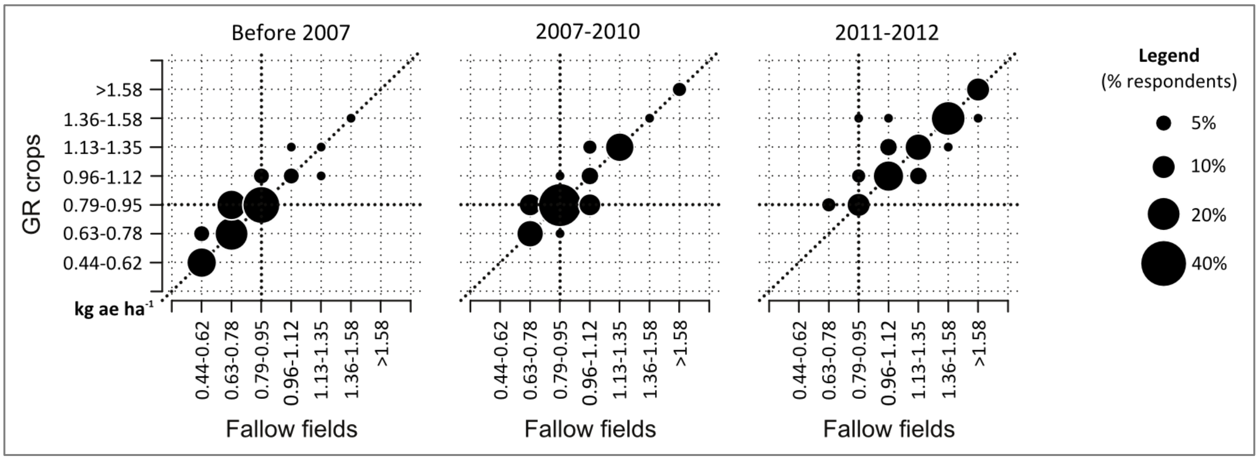


Figure 2.5 Glyphosate use rate in fallow fields in western Kansas plotted against that in GR fields during 2011-2012 reported by crop consultants (n=44, before 2007; n=47, 2007-2010; n=48, 2011-2012). Both axes are glyphosate use rate in kg ae ha^{-1} and circles represent percentage of respondents in proportion to the legend. The highlighted grids represent normal use rate of glyphosate.

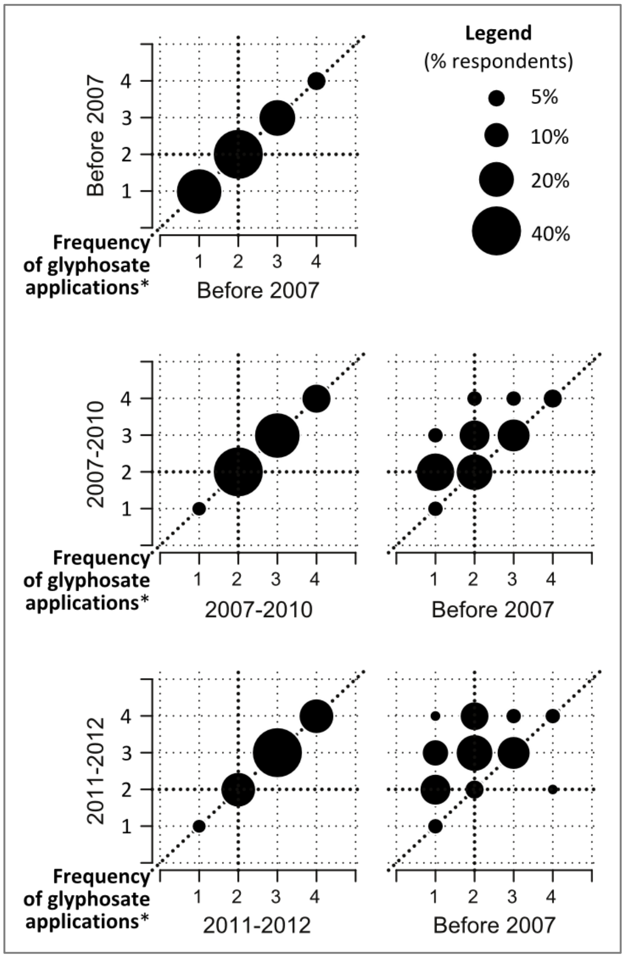


Figure 2.6 Glyphosate use frequency in fallow fields in western Kansas reported by crop consultants (n=47). Both axes are glyphosate use frequency per season and circles represent percentage of respondents in proportion to the legend**.** Circles above the diagonal line indicate increased glyphosate application frequency during the survey period in Y-axis compared to that during the survey period in X-axis.

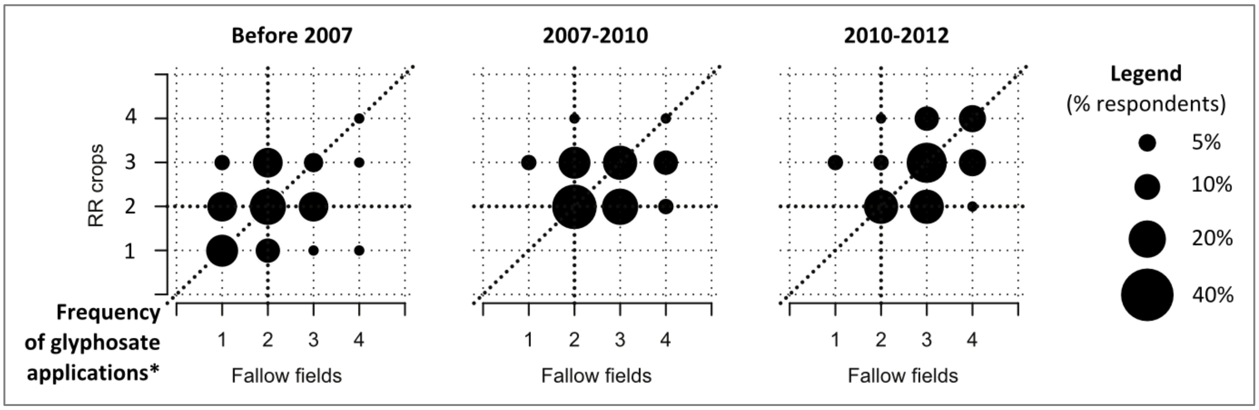


Figure 2.7 Glyphosate use frequency in fallow fields in western Kansas plotted against that in GR fields during 2011-2012 reported by crop consultants (n=47). Both axes are glyphosate use frequency per season and circles represent percentage of respondents in proportion to the legend. Circles above the diagonal line indicate increased glyphosate application frequency during the survey period in Y-axis compared to that during the survey period in X-axis.

*, glyphosate application frequency in a crop growing season

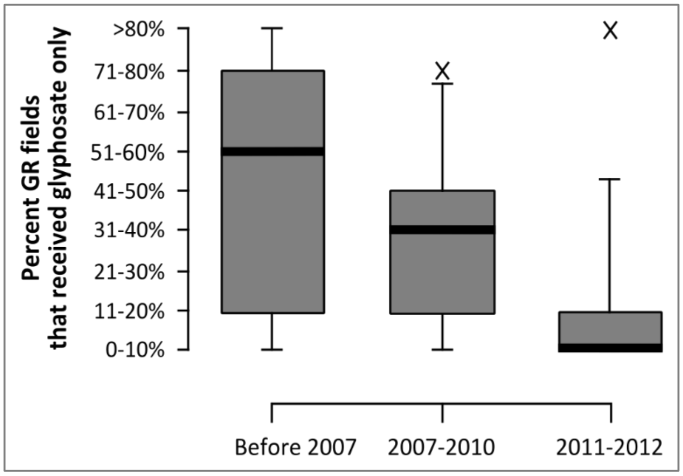


Figure 2.8 Box plots showing exclusive use of glyphosate for weed control in GR crops in western Kansas reported by crop consultants (n=42, before 2007; n=47, 2007-2010; n=52, 2011-2012). The box plots show the upper (Q3) and lower (Q1) quartiles and the median. The median is identified by a line inside the box. The length of the box represents the interquartile range (middle 50% of values). The vertical lines are the full range of values in the data except outliers. X indicates outlier response.

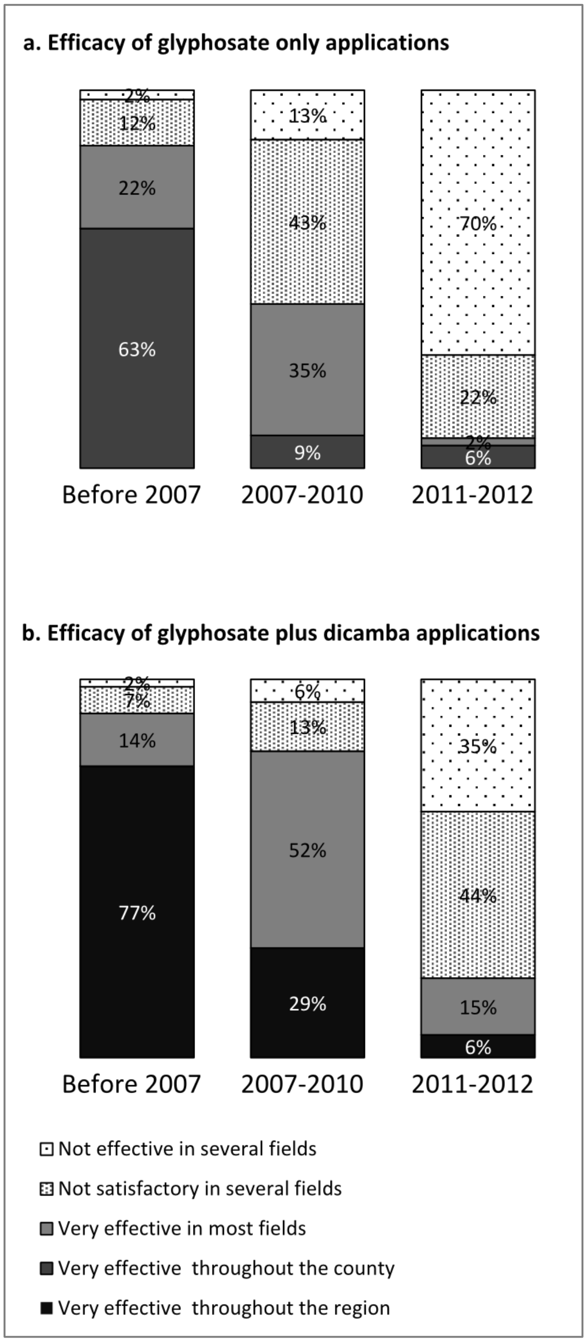


Figure 2.9 Efficacy of glyphosate with and without dicamba in controlling kochia reported by crop consultants. (a) Efficacy of glyphosate-only applications (n=41, before 2007; n=46, 2007-2010; n=50, 2011-2012), and (b) Efficacy of glyphosate plus >0.25 kg ae ha^{-1} dicamba applications (n=43, before 2007; n=48, 2007-2010; n=52, 2011-2012).

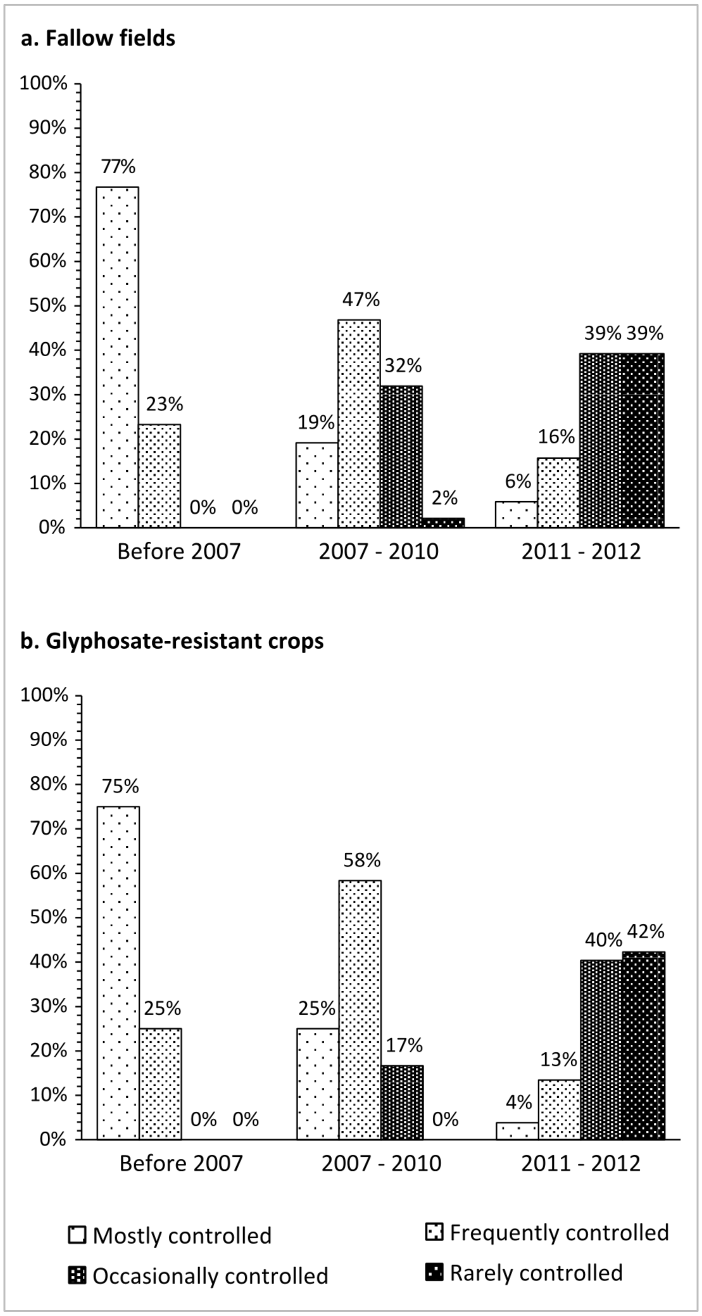


Figure 2.10 Difficulty in controlling kochia in western Kansas reported by crop consultants. (a) in fallow fields (n=43, before 2007; n=47, 2007-2010; n=51, 2011-2012), and (b) in GR crops (n=44, before 2007; n=48, 2007-2010; n=52, 2011-2012).

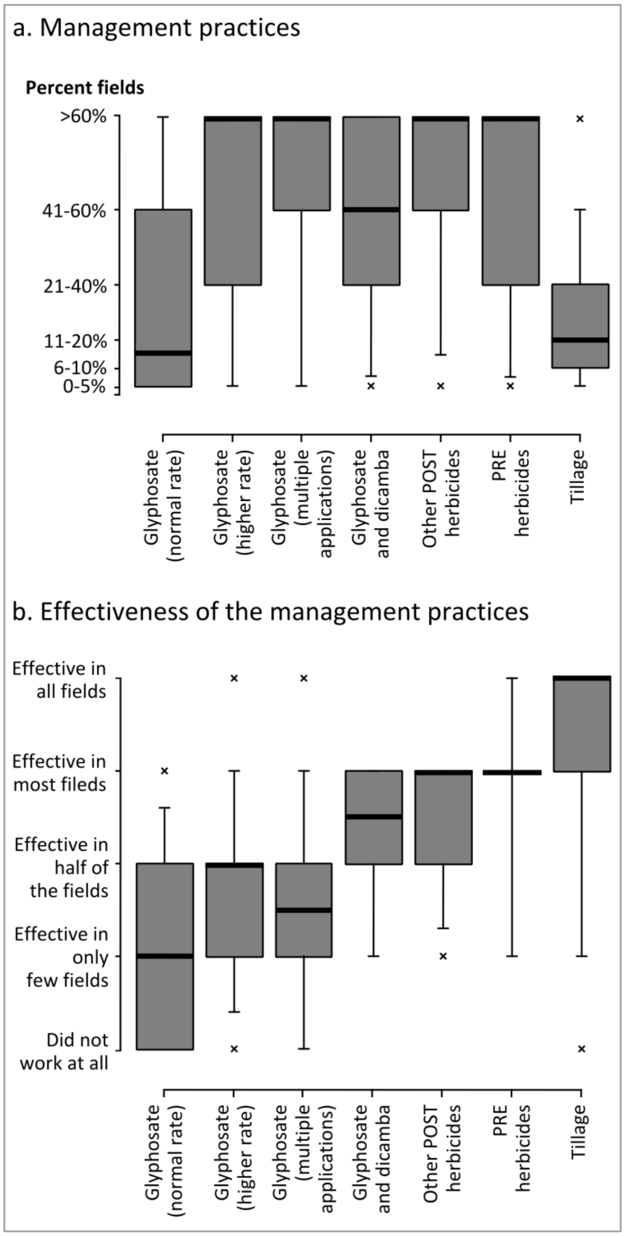


Figure 2.11 Management practices employed to control kochia during 2011-2012 and their effectiveness reported by crop consultants. (a) kochia management practices (n=48-52), and (b) effectiveness of kochia management practices (n=46-52). The box plots show the upper (Q3) and lower (Q1) quartiles and the median. The median is identified by a line inside the box. The length of the box represents the interquartile range (middle 50% of values). The vertical lines are the full range of values in the data except outliers. X indicates outlier response.

Chapter 3 - Differential Response of Kochia Populations to Glyphosate: Analysis from an Evolutionary Perspective

Abstract

Kochia [*Kochia scoparia* (L.) Schrad.] control failures with glyphosate were first reported in western Kansas during the mid-2000's. Four kochia populations in the region were confirmed resistant to glyphosate in 2007 and an additional eight populations were confirmed to be glyphosate-resistant (GR) in 2010. In 2012, 40 kochia populations, mostly from western Kansas, were evaluated for tolerance to glyphosate to ascertain the feasibility of detecting low level glyphosate resistance in kochia populations using *in vivo* leaf disc shikimate accumulation assay. Kochia populations showed varied response to 0.84 kg ae ha^{-1} glyphosate 18 d after treatment, ranging from 0 to 100% mortality. Most plants of populations from the western one-third of Kansas survived the glyphosate treatment. Many other populations including two of three populations from Oklahoma exhibited definitive levels of glyphosate resistance. Nearly one-half of the kochia populations from western Kansas and those from Idaho and South Dakota showed elevated tolerance to glyphosate. The results indicated that kochia populations were at different stages of evolved resistance to glyphosate. The amount of shikimate accumulated in glyphosate-treated (100 µM) leaf discs after 16 h was generally greater in resistant plants than in susceptible plants or plants with elevated tolerance to glyphosate. However, no clear distinction was observed among susceptible plants or those exhibiting greater than 50% injury. A better understanding of how kochia populations are evolving to glyphosate resistance is important to halt further evolution of glyphosate resistance and to preserve the value of glyphosate in crop production in the region.

Introduction

Glyphosate is the most widely used herbicide in global agriculture. Though the herbicide was first commercially available in 1974, its use in crop production systems was limited until the late 1900’s when use increased dramatically following introduction of Roundup Ready® crops. Extensive use of glyphosate has resulted in evolved resistance in many weed biotypes (Duke and Powles 2008, Powles 2008) and the impact is increasing at a rapid rate (Heap 2013).

Kochia [*Kochia scoparia* (L.) Schrad.] is an economically important weed of Central Great Plains agriculture. In addition to its adaptation in semiarid environments and conservation tillage practices, it is prone to evolve resistance to common herbicides used to control kochia (e.g., triazines in 1976; acetolactate synthase (ALS)-inhibitors in 1986; and dicamba in 1995) (Heap 2013, Foes et al. 1999, Peterson 1999). During the late 1990’s and early 2000’s, glyphosate became the most common herbicide in chemical fallow and glyphosate-resistant (GR) crops. During those years, glyphosate, even at reduced rates, provided superior and economical weed control solutions in many situations in the region.

Kochia control failures with glyphosate were first reported in southwest Kansas during the mid-2000’s. Monsanto Company (St. Louis, MO.) found elevated tolerance in some kochia populations, but not at distinctive levels to qualify as resistance (personal communication with J. M. Fenderson). Reduced rates of glyphosate (<0.84 kg ae ha^{-1}) being used in the region were thought to be the primary driver for selection of elevated tolerance in those populations. However, many segregating progenies of a population from Thomas County, KS (Chapter 1) and three other populations from western Kansas collected in 2007 (Waite et al. 2013) were found to survive higher-than-normal use rates of glyphosate. Those were the first documented cases of confirmed glyphosate resistance in kochia. In the following years, reports of kochia control failures with glyphosate gradually increased and by 2010 glyphosate resistance was confirmed in an additional eight widely dispersed kochia populations from southwest to north-central Kansas (Chapter 1). Moreover, in a recent survey of crop advisors, more than half of the respondents reported GR kochia in at least 50% of fields in western Kansas and almost all reported at least some level of presence of GR kochia (Chapter 2).

The underlying molecular mechanism that confers glyphosate resistance in kochia populations found in the northern Great Plains has been suggested by Wiersma (2012), although, the evolutionary course of glyphosate resistance in kochia populations is lacking. Varying levels of resistance from low to moderate were observed in populations that were characterized for glyphosate resistance (Chapter 1). Whether the resistance is very low in the early stage of evolution and increases in the successive generations is not clear. From this perspective, early detection of increasing tolerance to glyphosate may be crucial for halting further escalation, primarily in the region where resistance has not yet been confirmed.

Shikimate (shikimic acid) accumulates in plant tissues upon exposure to glyphosate treatment. Depending on the glyphosate dose used, the amount of shikimate accumulated is usually greater in susceptible than in resistant plants (Henry et al. 2007, Mueller et al. 2008). Shikimate accumulation assay is widely used alone or in conjunction with other methods to confirm evolved glyphosate resistance in weed populations (Chapter 1, Gaines et al. 2012, Wiersma 2012). An *in vivo* shikimate accumulation test described by Shaner et al. (2005) allows efficient detection of glyphosate resistance in plant species (Koger et al. 2005).

In this study, 40 kochia populations from throughout the central Great Plains, mostly from western Kansas but also Idaho, Oklahoma, and South Dakota, were evaluated for resistance to glyphosate by treating plants with a recommended dose of glyphosate (0.84 kg ae ha^{-1}). In addition, the feasibility of detecting low level resistance to glyphosate in kochia populations was assessed using *in vivo* shikimate accumulation test. Together with the prior findings, results from this study are discussed from an evolutionary perspective.

Materials and Methods

Seed Collection

Seed were collected from 40 fields in fall 2012. The collections include western Kansas (34 populations), Idaho (1 population) northwest Oklahoma (3 populations), and eastern South Dakota (2 populations) (Table 3.1). Seed were harvested from 10 to 20 mature plants per field with unknown cropping history and bulked for each population. Geographic locations were recorded using GPS coordinates for each site with ≤25 m deviation from seed source. All Kansas populations were subcategorized into six crop districts as designated by the National Agricultural Statistics Service (Figure 3.1). Clean seed were stored in plastic zip lock (Zipper) bags at normal room temperature for 6 to 8 weeks before testing.

Raising Seedlings and Glyphosate Application

Approximately 300 seeds of each population were sown in 50-cm by 25-cm by 5-cm plastic trays containing 4-cm deep commercial potting mix (Miracle-Gro, Scotts Miracle-Gro Products Inc., Marysville, OH) in a greenhouse. Thirty-six two-week old seedlings (1 to 1.5 cm tall) from each population were transplanted into 7-cm by 3.5-cm by 8-cm plastic cells. Remaining seedlings in the original trays were thinned to 36 seedlings per tray maintaining uniform plant to plant spacing and plant size. Plants were grown under 25/20 (±2) C day/night temperature with 15/9 h day/night photoperiod, supplemented with 200 µmol $m^{-2}s^{-1}$ illumination provided with sodium vapors lamps. Plants were regularly watered as needed and fertilized every two weeks. Ten to 12-cm tall plants were sprayed with potassium salt of glyphosate (Roundup Weathermax®, Monsanto Company, St. Louis, MO) at 0.84 kg ae ha^{-1} with a moving single-nozzle bench-type sprayer (Research Track Sprayer, De Vries Manufacturing, RR 1 Box 184, Hollandale, MN) equipped with a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., P.O. Box 7900, Wheaton, IL) delivering 168 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. Glyphosate treatment included ammonium sulfate (AMS) at 2% w/v.

After analyzing the response (mortality, injury, and biomass) of all forty populations to 0.84 kg ae ha^{-1} rate of glyphosate, the following populations, Pratt, Barton, Oklahoma3, and Scott10012, were sown in 20-cm by10-cm by 10-cm plastic pots for evaluating response to higher rate(s) of glyphosate. Individual seedlings were transplanted into 6-cm by 6-cm by 6-cm plastic pots and grown similarly as described above. Plants were treated with glyphosate rates of 0.84 (Pratt), 1.68 (Barton, Oklahoma3, and Scott10012), and 3.36 (Oklahoma3 and Scott10012) kg ae ha^{-1} using the same procedure as previously described.

Plant Injury Evaluation, Fresh Weight Measurement, and Categorization of Populations

Plants were assessed for injury and aboveground fresh biomass 18 d after treatment (DAT). Plant tolerance was assessed based on composite visual estimations of aboveground growth inhibition and foliar injury or plant health (chlorosis and necrosis) using a scale of 0 (no effect) to 100 (plant death). Aboveground plants parts with 0 to 30% injury were harvested in bulk within population and weighed immediately (fresh biomass), followed by plants with 31-60, 61-

90, 91-99, and 100% injury levels. Fresh biomass, instead of dry biomass, was taken to account for clear difference between dead and alive plants, especially with severe injury. Injury quotient for each population was calculated as

$$\text{Injury quotient} = \sum_{i=A}^{5} \frac{n_i}{100}\left(1 + \frac{\bar{x}_i}{100}\right) \qquad \text{Eq. 3.1}$$

where n represents frequency of individuals and $\bar{x}$ represents mid-values for injury category i. Overall efficacy of glyphosate was calculated as

$$\text{Overall efficacy} = 100 - \text{Injury quotient}$$

Populations were ranked based on injury quotient (1 = least tolerant and 40 = most tolerant) and aboveground fresh biomass (1 = greatest biomass and 40 = least biomass). Since a strong correlation was observed between two ranks ($R^2 = 0.935$) (Figure 3.2), populations were categorized (Figure 3.3; Table 3.1) based on injury quotient. Considering similarity in population composition, populations that ranked from 1 to 5, 6 to 15, 16 to 25, 26 to 35, and 36 to 40 were grouped arbitrarily into five categories, A, B, C, D, and E, respectively.

Mortality (percent of populations) for Pratt, Barton, Oklahoma3, and Scott10012 populations was assessed 21 DAT. Plants with any green tissue were considered alive.

Shikimate Accumulation Assay

Before applying glyphosate, a shikimate accumulation assay (Shaner et al. 2005) was conducted on nine randomly selected plants from each of the forty populations (total number of plants = 360). The selected plants were labeled with numbers for injury evaluations at 18 d after 0.84 kg ae ha^{-1} glyphosate application. Four 5-mm leaf discs were excised from the two youngest fully-expanded leaves from each plant. Three leaf discs from each plant were placed in transparent 96-

well microtiter plates containing 100 µL of 100 µM of glyphosate (Glyphosate PESTANAL®, analytical standard, Sigma-Aldrich Co. LLC, St. Louis, MO) solution and one leaf disc placed in 100 µL buffer (Shaner et al. 2005) without glyphosate. The plates were immediately wrapped with clear plastic to reduce evaporation and incubated at room temperature for 16 h under continuous light (200 µmol $m^{-2}s^{-1}$). The plates were frozen at -20 C for 20 min and thawed at 60 C for 20 min. Twenty-five microliters of 1.25 N HCl was added to each well, and plates were covered and incubated at 60 C until the entire leaf disc turned brown (approx. 20 to 25 min). Twenty-five microliters of aliquot from each wells was transferred to corresponding wells in a new plate and was oxidized by adding 100 µL of 0.25% periodic acid 0.25% sodium-m-periodate solution and incubating at 40 C for 20 min. Known concentrations of shikimate solutions were similarly oxidized for generating a standard curve. After oxidation, the solution was quenched by adding 100 µL of 0.6 M NaOH/0.22 M Na_2SO_3 solution to each well. Optical density was measured within three to five minutes using a spectrophotometer (Epoch Micro-Volume Spectrophotometer System, BioTek, Winooski, VT) at 380 nm. Optical density (OD380) values were converted to shikimate concentration (ng μL^{-1}) by using the standard curve. The amount of shikimate extracted was calculated by subtracting the shikimate concentration in corresponding control (0 µM glyphosate) wells. The ng μL^{-1} shikimate values were further converted to ng shikimate per mm^2 of leaf disc (ng mm^{-2} leaf disc).

Statistical Analysis

Injury quotient and aboveground fresh biomass data grouped by population categories were subjected to analysis of variance (ANOVA) using *aov()* function in R 2.15.2 (R Development Core Team 2013). The means for injury quotient and biomass were compared using Tukey’s

HSD test with confidence level at 0.95. Correlation analyses among mortality, overall efficacy, and frequency of individuals with ≤30% injury were performed using R 2.15.2.

Results

Overall Efficacy, Fresh Weight Biomass, and Characterization of Population Categories

Overall efficacy of glyphosate (0.84 kg ae ha^{-1}) on kochia populations 18 DAT ranged from 17 to 100%. Mean efficacy was 100, 92, 77, 49, 25% for categories A, B, C, D, and E, respectively. Aboveground fresh biomass ranged from 0.15 to 5.52 g per plant (Figure 3.3) and were strongly correlated with the injury quotient ($r=0.97$, $P<0.01$).

A general characterization of population categories is shown in Table 3.1. Most plants from category A populations did not survive 0.84 kg ae ha^{-1} glyphosate (Figure 3.4). Only a few plants from three populations (Ellis246, Republic, and Russell250) survived, but with >90% injury. In category B populations, few to nearly one-third of the plants survived with <90% injury (Figure 3.5). While plants with ≤30% injury were absent in most populations, up to 5% of plants with ≤30% injury were present in some populations. A majority of plants showed ≥60% injury in category C populations (Figure 3.6). The other plants showed ≤30 or 31-60% injury in inconsistent proportions among the populations. In category D populations, a majority of plants survived with ≤60% injury with significant number of plants showing ≤30% injury (Figure 3.7). Most plants in category E populations showed ≤60% injury, with at least half of them showing ≤30% injury (Figure 3.8).

Spatial Distribution of Populations with Respect to Population Categories

Populations from the North Central Kansas crop districts ranked within either category A or B (Table 3.1). Central district populations ranked in A, B or C categories. All populations from Southwest district fell into C or D categories. West Central and South Central district populations showed diverse response. One population (Oklahoma3) from Oklahoma fell into category E and all the other non-Kansas populations belonged to A, B, or C categories. Populations from the Northwest district fell into D or E categories.

Glyphosate Efficacy and Population Composition

Relationships among mortality, overall glyphosate efficacy, and frequency of plants with ≤30% injury are shown in Figure 3.9. A strong positive correlation was observed between overall efficacy of glyphosate and percent mortality ($r=0.95$, $P<0.001$) (Figure 3.9a). Percent mortality was negatively correlated with frequency of individuals with ≤30 injury ($r=-0.81$, $P<0.01$) (Figure 3.9b). Nearly a perfect reverse relationship was observed between frequency of plants with ≤30% injury and overall efficacy ($r=-0.95$, $P=0$) (Figure 3.9c).

Response of Selected Kochia Populations to Glyphosate Rates

Except for Pratt, most plants from Barton, Oklahoma3, and Scott10012 populations survived 0.84 kg ae ha^{-1} of glyphosate (Table 3.2). However, none of the plants from the Barton population survived 1.68 kg ae ha^{-1}. Doubling of glyphosate rate from 0.84 to 1.68 kg ae ha^{-1} dramatically reduced the percentage of surviving plants from Oklahoma3 and Scott10012 populations. None of the plants from Oklahoma3 and only 2 of 24 plants from the Scott10012 population survived 3.36 kg ae ha^{-1} of glyphosate.

Shikimate Accumulation and Visual Injury

In general, plants exhibiting greater injury accumulated more shikimate in leaf discs than lesser injured plants ($R^2=0.79$, $P<0.01$) (data not shown). Shikimate accumulations in nine randomly selected plants per population with respect to injury 18 days after 0.84 kg ae ha^{-1} glyphosate treatment are shown in Figures 3.4, 3.5, 3.6, 3.7, and 3.8 for populations belonging to categories A, B, C, D, and E, respectively. For each population, shikimate accumulation was generally higher for plants with greater injury. Histograms of shikimate accumulation clustered by plant injury levels are shown in Figure 3.10. Although shikimate accumulation increased with increasing plant injury level, a significant overlap between the adjacent injury ranges was evident. Plants exhibiting little or no injury consistently accumulated ≤40 ng mm^{-2} leaf disc shikimate. Generally, plants with <50% injury accumulated ≤60 ng mm^{-2} leaf disc shikimate and those with >50% injury accumulated >60 ng mm^{-2} leaf disc shikimate. While shikimate accumulation >140 ng mm^{-2} leaf disc was observed in only those plants that did not survive the glyphosate treatment, at least half of those plants accumulated 60 to 140 ng shikimate mm^{-2} leaf disc.

Discussion

This study showed variability of kochia response to 0.84 kg ae ha^{-1} of glyphosate both at individual and population levels. Previous study (Chapter 1) demonstrated that less than half the rate of glyphosate used was phytotoxic to the glyphosate-susceptible kochia population. All plants from only 3 of 40 populations analyzed in this study were killed. In another study, susceptibility of kochia plants to glyphosate increased under greenhouse conditions compared to outdoor conditions (Chapter 1), thus, survival in this study likely would have been higher under field conditions. Though a few plants of some populations in categories A and B survived, most surviving plants were severely injured (average efficacy >92%). Depending on the weed management goal and glyphosate use rate, such situations may not have practical impact at field level.

A higher proportion of plants from category C populations compared to category A populations survived the glyphosate treatment. Under field conditions, efficacy of glyphosate on those populations would likely be unacceptable (average efficacy = 72%). More definitive evidence of glyphosate resistance was observed in kochia populations in categories D and E (total of 15 populations) with average glyphosate efficacy of 49 and 25%, respectively. Those populations were mostly from Northwest, Southwest, or West-Central Kansas crop districts, indicating greater prevalence of glyphosate-resistant populations in the western one-third of Kansas. One each of three Oklahoma populations was among the C, D, and E categories. Results from this study were fairly consistent with results of the online survey of crop advisors conducted in fall 2012 (Chapter 2). In that survey, almost all survey respondents reported presence of glyphosate-resistant kochia, and about half reported glyphosate-resistant kochia in more than 50% of fields. Thus, it is estimated that at least one-third of kochia populations in western Kansas have evolved resistance to glyphosate to a level at which the recommended field use rate of glyphosate is not effective.

Relationships among mortality, overall efficacy, and frequency of plants with ≤30% injury as shown in Figure 3.9 indicated that populations evolved towards greater levels of tolerance both at individual plant and population levels. Response of the selected populations to higher rates of glyphosate further suggests the same. This may be attributed to changes in allele frequency (the allele conferring glyphosate resistance) in populations not only by increased number of individuals carrying the alleles, but also by increased number of alleles in individual plants. It is reasonable to assume that the mean of the phenotype (susceptibility to glyphosate) would be similar in all populations prior to the use of glyphosate in the region. Though kochia's response to glyphosate was not measured in a single population over several generations of selection pressure, the overall pattern indicates that populations are under directional selection with shifts in both range and average phenotypic values. Based on these results, an illustrative population composition for each category is shown in Figure 3.11.

Wiersma (2012) found amplified EPSPS gene copies in all northern Great Plains GR kochia populations and suggested that the level of resistance is strongly related to the EPSPS gene copy number. This indicates that each amplified copy is fully functional (not pseudo copies) and contributes equally to the phenotypes. The EPSPS gene copy number in resistant populations ranged from three to nine relative to the number of ALS gene. As the selection appears to be directional, it can be concluded that more EPSPS gene copies are accumulated in individuals under selection over time.

Furthermore, Wiersma (2012) found a kochia population initially thought to be susceptible to glyphosate was segregating for EPSPS gene copies. In this population, only 2 of 16 plants had three EPSPS:ALS relative gene copies and another two had 1.5 copies. While complete mortality was achieved at 1.68 kg ae ha^{-1} of glyphosate for that population, a six-times lower rate (0.28 kg ae ha^{-1}) caused >80% mortality. These results clearly indicate that evolving resistance may not be realized for a few generations until the populations evolve to practical field level resistance. By the time populations reach that level, most individuals in the population have three or more copies of EPSPS gene. Herbicide resistance is usually not diagnosed until the population exhibits control failures or distinctive levels of resistance (Baucom and Mauricio 2010). Herbicide resistance studies often ignore populations that show low level resistance. Indeed, several suspected glyphosate-resistant kochia populations collected from the region in 2010 showed marginally elevated tolerance and were not tested further (Chapter 1). This could be the possible reason why three or more EPSPS:ALS relative gene copies are consistently found in resistant populations under investigation. A point to note here is that the actual fold change in EPSPS copy number relative to susceptible counterpart is 40 to 45% greater compared to EPSPS:ALS relative copy number. This value is approximately 0.7 for susceptible plants (Wiersma 2012).

It can be speculated that reduced use rates of glyphosate may have favored establishing the initial EPSPS gene amplification/duplication event in the populations by allowing individuals with low number of EPSPS genes to survive and reproduce. Subsequent higher use rates and application frequencies may have facilitated the evolution to higher levels of resistance. The average glyphosate use rate in western Kansas (as reported by crop advisors) increased by 50% (from 0.8 to 1.2 kg ae ha^{-1}) from before 2007 through 2012 (Chapter 2). Correspondingly, reports of poor kochia control with glyphosate in western Kansas emerged before 2007 and were profuse by 2012 (Chapter 1 and 2). Since as little as half of the recommended dose of glyphosate effectively controls susceptible kochia populations, twice the normal rate would provide similar efficacy on populations having up to 4-fold higher EPSPS gene copies. While other mechanisms are possible, it is likely that a small fraction of individuals in category A and B populations contain amplified EPSPS gene copies at low number. Glyphosate rates should not be compromised in this case as it will likely promote establishment of the alleles in the population. Highly effective rates of glyphosate or other methods for few generations may cause a breakpoint preventing populations from transitioning to category D or E.

The exact mechanism how EPSPS gene is amplified is unknown. Gaines et al. (2010) and Wiersma (2012) discussed some of the possible mechanisms in GR Palmer amaranth (*Amaranthus palmeri*) and kochia, respectively. These include DNA-mediated gene amplification mechanisms or some other forms of chromosomal rearrangements. A common origin of EPSPS gene amplification event and subsequent dispersion is less likely considering the presence of glyphosate resistance in widely dispersed populations (from northern Oklahoma to southern Canada). It is more convincing to suspect that several GR kochia populations in the region evolved independently and donated alleles to other populations in the vicinity. In different events, the duplicated or amplified chromosomal fragment may not necessarily reside at the same chromosomal location. As a result, regardless of the mechanism, populations may carry the extra gene copies in varying locations on the chromosome(s). Rapid gene flow in kochia is possible by seed dispersal (Fay et al. 1992) and cross pollination (Stallings et al. 1995). Thus, one of the possibilities is that the additional increase in extra alleles is through unidirectional or bidirectional gene flow. It may not be mere coincidence that herbicide resistance due to gene amplification has been documented in a dioecious species such as Palmer amaranth (Gaines et al. 2010), a strictly cross-pollinating species such as Italian ryegrass (Salas et al. 2012), and now in kochia.

Results from this study showed a fairly strong correlation between shikimate accumulation in leaf discs and levels of resistance. However, shikimate accumulation results may be confounding, especially when the level of resistance is very low. While the *in vivo* shikimate accumulation is more efficient, it may not be as precise as the HPLC method (Shaner 2010). Moreover, genetic (Mengistu and Messersmith 2002) and morphological variation is common in kochia populations. Possible variations in number of cells and chlorophyll content in leaf discs among and within the populations might have affected total shikimate accumulation in leaf discs. Additionally, amount of shikimate extracted from leaf discs may depend on leaf thickness. These possible factors were not measured in this study. Nevertheless, *in vivo* leaf disc shikimate accumulation test can be very useful in large-scale screening of GR kochia populations in resistance evolution, inheritance, and monitoring studies.

Kochia populations studied exhibited varied response to glyphosate. Results suggest that these populations are at different stages of evolved resistance to glyphosate. From a scientific point of view, all the populations within categories B or higher can be classified as GR populations, but a majority of the populations are likely to be effectively controlled with normal or higher-than-normal rates of glyphosate. However, it appears that when the population is under selection, a certain level of resistance to glyphosate in the population is transient. Greater understanding of selection process is important. As of 2012, it is estimated that at least one-third of western Kansas kochia populations have evolved resistance to the recommended rate of glyphosate, and this finding is in agreement with survey results (Chapter 2). Presence of glyphosate-resistant kochia populations have recently been reported in five other central Great Plains states and Alberta Canada (Heap 2013). In addition, two of three Oklahoma populations showed resistance to glyphosate. Definitive answers to the question whether the widespread occurrence of resistance is due to multiple independent origins and the outcome of inter-population crossing between evolving populations remain unknown. A better understanding of the underlying processes of evolution, inheritance, and spread of the resistance is important to sustain the utility of glyphosate in central Great Plains agriculture.

References

Baucom, R. S. and R. Mauricio. 2010. Defence against the herbicide RoundUp® predates its widespread use. Evol. Ecol. Res. 12:131-141.

Duke, S. O. and S. B. Powles. 2008. Glyphosate: a once-in-a-century herbicide. Pest Manag. Sci. 64: 319-325.

Fay, P. K., D. M. Mulugeta, and W. E. Dyer. 1992. The role of seed dispersal in the spread of sulfonylurea-resistant *Kochia scoparia*. Weed Sci. Soc. Am. Abstr. 32:49. Weed Science Society of America, Lawrence, KS.

Foes, M. J., L. Liu, G. Vigue, E. W. Stoller, L. M. Wax, and P. J. Tranel. 1999. A kochia (*Kochia scoparia*) biotype resistant to triazine and ALS-inhibiting herbicides. Weed Sci. 47:21-27.

Gaines, T. A., W. Zhang, D. Wang, B. Bukun, S. T. Chisholm, D. L. Shaner, S. J. Nissen, W. L. Patzoldt, P. J. Tranel, A. S. Culpepper, T. L. Grey, T. M. Webster, W. K. Vencill, R. D. Sammons, J. Jiang, C. Preston, J. E. Leach, and P. Westra. 2010. Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. PNAS. 107:1029-1034.

Heap, I. The International Survey of Herbicide Resistant Weeds. Online. Internet. Accessed June 11, 2013. Available www.weedscience.com

Henry, W. B., D. L. Shaner, and M. S. West. 2007. Shikimate accumulation in sunflower, wheat, and proso millet after glyphosate application. Weed Sci. 55:1-5.

Koger, C. H., D. L. Shaner, W. B. Henry, T. Nadler-Hassar, W. E. Thomas, and J. W. Wilcut. 2005. Assessment of two nondestructive assays for detecting glyphosate resistance in horseweed (*Conyza canadensis*). Weed Sci. 53:438-445.

Mengistu, L. W. and C. G. Messersmith. 2002. Genetic diversity of kochia. Weed Sci. 50:4 498-503.

Muller, T., A. Tellis, J. E. Beeler, S. D. Sharma, and M. Singh. 2008. Shikimate accumulation in nine weedy species following glyphosate application. Weed Res. 48:455-460.

Peterson, D. E. 1999. The Impact of herbicide-resistant weeds on Kansas agriculture. Weed Technol. 13:632-635.

Powles, S. B. 2008. Evolved glyphosate-resistant weeds around the world: lessons to be learnt. Pest Manag. Sci. 64:360-365.

Salas, R. A., F. E. Dayan, Z. Pan, S. B. Watson, J. W. Dickson, R. C. Scott, and N. R. Burgos. EPSPS gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) from Arkansas. Pest Manag. Sci. 68:1223-1230.

Shaner, D. L. 2010. Testing methods for glyphosate resistance. In: Glyphosate resistance in crops and weeds: History, development, and management. Ed Nandula, V. K. John Wiley & Sons, Inc. pp 93-111.

Shaner, D. L., T. Nadler-Hasser, and C. H. Koger. 2005. A rapid in vivo shikimate accumulation assay with excised leaf discs. Weed Sci. 53:769-774.

Stallings, G. P., D. C. Thill, C. A. Mallory-Smith, and B. Shafii. 1995. Pollen-mediated gene flow of sulfonylurea-resistant kochia (*Kochia scoparia*). Weed Sci. 43:95-102.

Wiersma, A. 2012. Regional whole plant and molecular response of Kochia scoparia to glyphosate. M.S. thesis. Fort Collins, Co. Colorado State University. 63 p.

Table 3.1 Kochia populations and geographic regions. Populations are sorted by categories.

Population	GPS coordinates		US	KS Crop district[b]	County	Category[a]	Remarks
	Latitude	Longitude					
Ellis246	38.9433	-099.4809	KS	C	Ellis	A	Most plants showed 100% injury
Phillips247	39.6838	-099.2390	KS	NC	Phillips	A	
Pratt	37.6005	-098.6480	KS	SC	Pratt	A	
Republic	39.7839	-097.6526	KS	NC	Republic	A	
Russell250	39.0522	-098.5130	KS	C	Russell	A	
Ellis12	-	-	KS	C	Ellis	B	Most plants showed ≥90% injury
Idaho	-	-	ID	-	-	B	
Logan	39.1024	-101.0350	KS	WC	Logan	B	
Ness240	38.6402	-099.7781	KS	WC	Ness	B	
Osborne	39.5453	-098.5433	KS	NC	Osborne	B	
Rice	38.3913	-098.2052	KS	C	Rice	B	
Rooks	39.2630	-099.1039	KS	NC	Rooks	B	
South Dakota2	44.3029	-96.9259	SD	-	Brookings	B	
Wallace	38.8931	-101.7970	KS	WC	Wallace	B	
Wichita	38.4809	-101.4140	KS	WC	Wichita	B	
Barton	38.5068	-098.8492	KS	C	Barton	C	Majority of plants showed ≥60% injury
Finney	38.0000	-100.9390	KS	SW	Finney	C	
Ford	37.9133	-099.8937	KS	SW	Ford	C	
Haskell	37.4968	-100.7810	KS	SW	Haskell	C	
Meade	37.2857	-100.1860	KS	SW	Meade	C	
Ness257	38.3355	-099.8980	KS	WC	Ness	C	
Oklahoma1	36.8091	-101.3522	OK	-	Texas	C	
Russell212	38.8606	-098.8168	KS	C	Russell	C	
Russell253	38.8121	-098.7925	KS	C	Russell	C	
South Dakota1	44.3176	-96.8495	SD	-	Brookings	C	
Decatur	39.9200	-100.5950	KS	NW	Decatur	D	Majority of plants showed ≤60% injury
Edwards216	37.9999	-099.2200	KS	SC	Edwards	D	
Gray	37.9178	-100.5010	KS	SW	Gray	D	
Greeley	38.3843	-101.7900	KS	WC	Greeley	D	
Norton221	39.6682	-100.0540	KS	NW	Norton	D	
Oklahoma2	36.5897	-101.5986	KS	-	Texas	D	
Rawlins	39.8173	-101.3350	KS	NW	Rawlins	D	
Scott237	-	-	KS	WC	Scott	D	
Sheridan	39.1692	-100.2390	KS	NW	Sheridan	D	
Stevens	37.3441	-101.1760	KS	SW	Steven	D	
Edwards219	37.9958	-099.2180	KS	SC	Edwards	E	Most plants showed ≤60% injury
Lane239	-	-	KS	WC	Lane239	E	
Oklahoma3	36.8500	-102.2277	OK	-	Texas	E	
Scott10012	-	-	KS	WC	Scott	E	
Thomas	39.4669	-100.9840	KS	NW	Thomas	E	

[a] categories based on injury quotient (Equation 3.1; Figure 3.2)

[b] Kansas crop districts as deginated by National Agricultural Statistics Service, refer to Figure 3.1.

Abbreviations: C, Central; NC, North Central; NW, Northwest; SC, South Central; SW, Southwest; WC, West Central

Table 3.2 Percent survival of selected kochia populations to increasing glyphosate rates at 18 d after treatment (n represents number of plants evaluated).

	Glyphosate rate[a]		
Population	0.84 kg ae ha^{-1}	1.68 kg ae ha^{-1}	3.36 kg ae ha^{-1}
	-------------- % survival 21 DAT---------------		
Pratt	0 (n=114)	-	-
Barton	70 (n=72)	0 (n=42)	-
Scott10012	98.5 (n=72)	17.5 (n=42)	8.3 (n=24)
Oklahoma3	100 (n=72)	20 (n=42)	0 (n=24)

[a] Glyphoate was applied to12 to 18 cm tall plants.

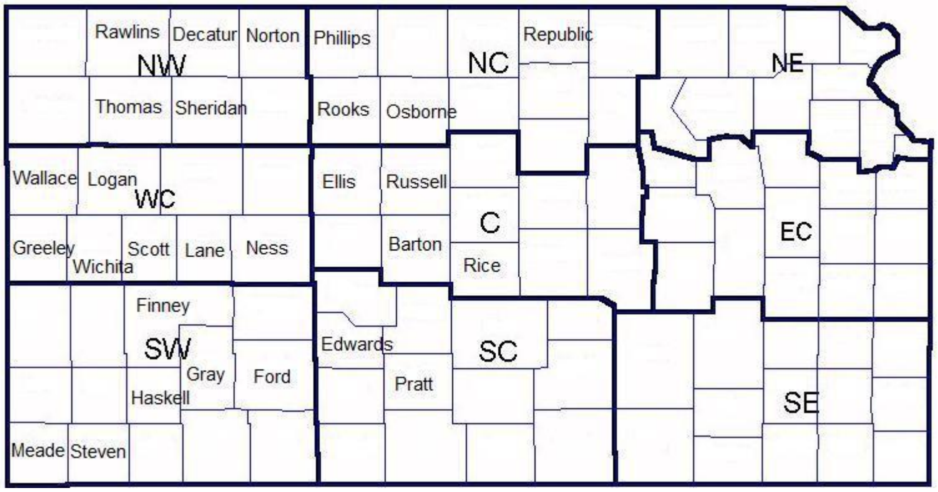


Figure 3.1 Individual western Kansas counties where kochia seed were collected in fall 2012. Kansas crop districts are designated by the National Agricultural Statistics Service (C, Central; NC, North Central; NW, Northwest; SC, South Central; SW, Southwest; WC, West Central).

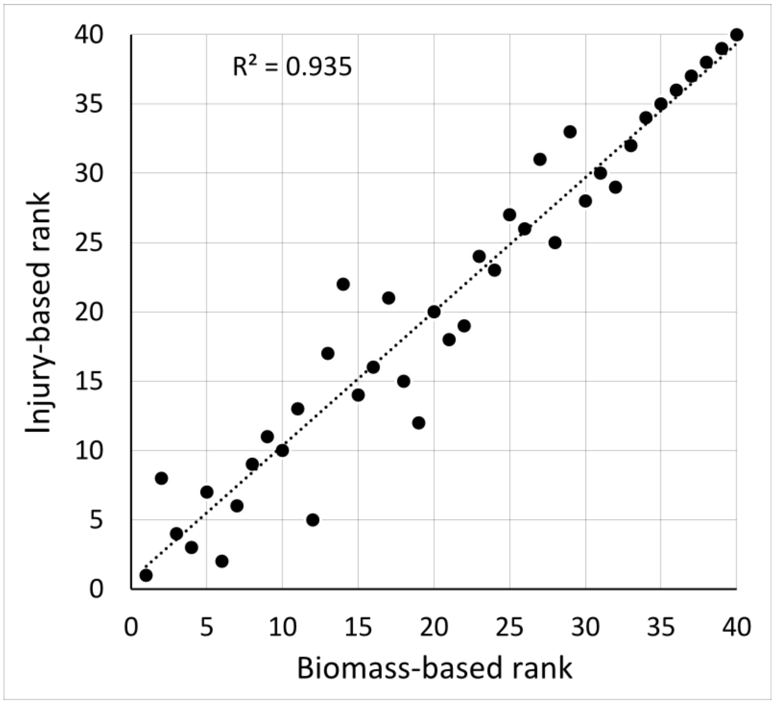


Figure 3.2 Correlation between visual injury-based and aboveground fresh weight biomass-based ranks of kochia populations treated with 0.84 kg ae ha^{-1} glyphosate.

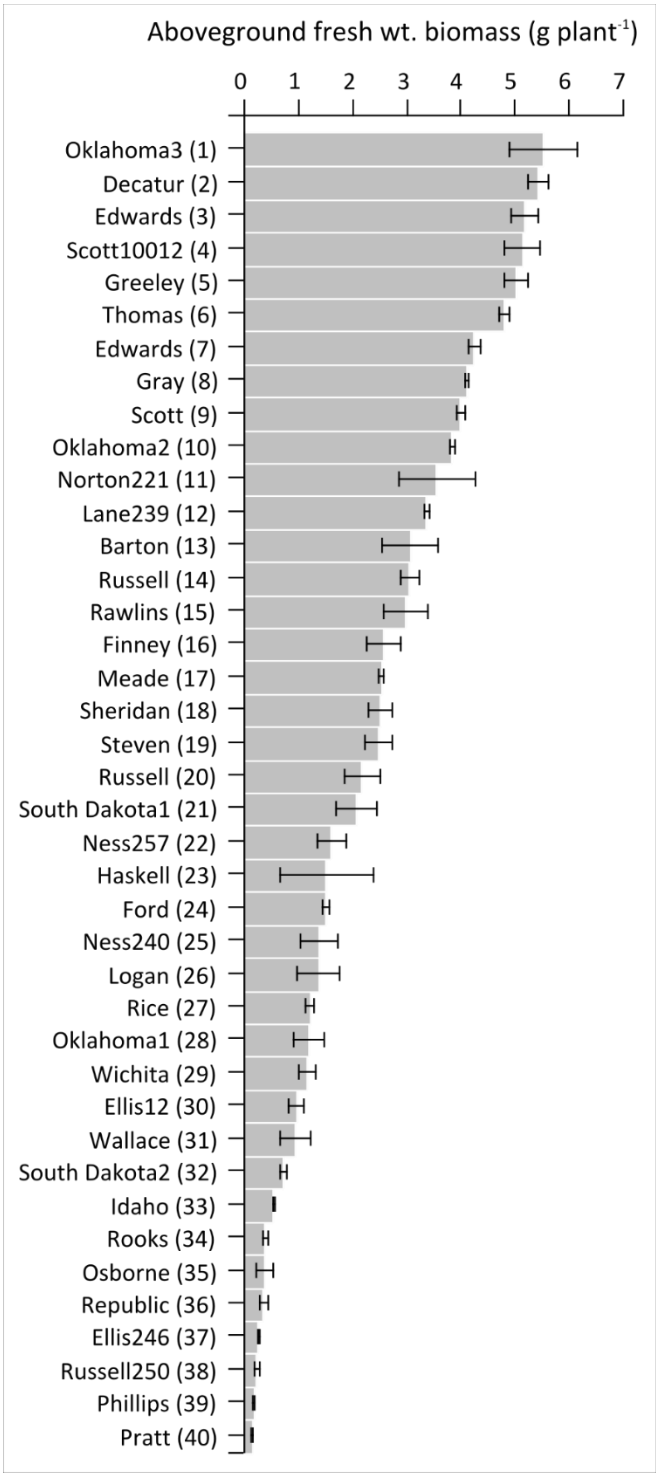


Figure 3.3 Aboveground fresh biomass (g plant^{-1}, n=72) of kochia populations measured 18 days after glyphosate treatment (0.84 kg ae ha^{-1}). Values in parentheses on vertical axis represent biomass-based rank as shown in Figure 3.2.

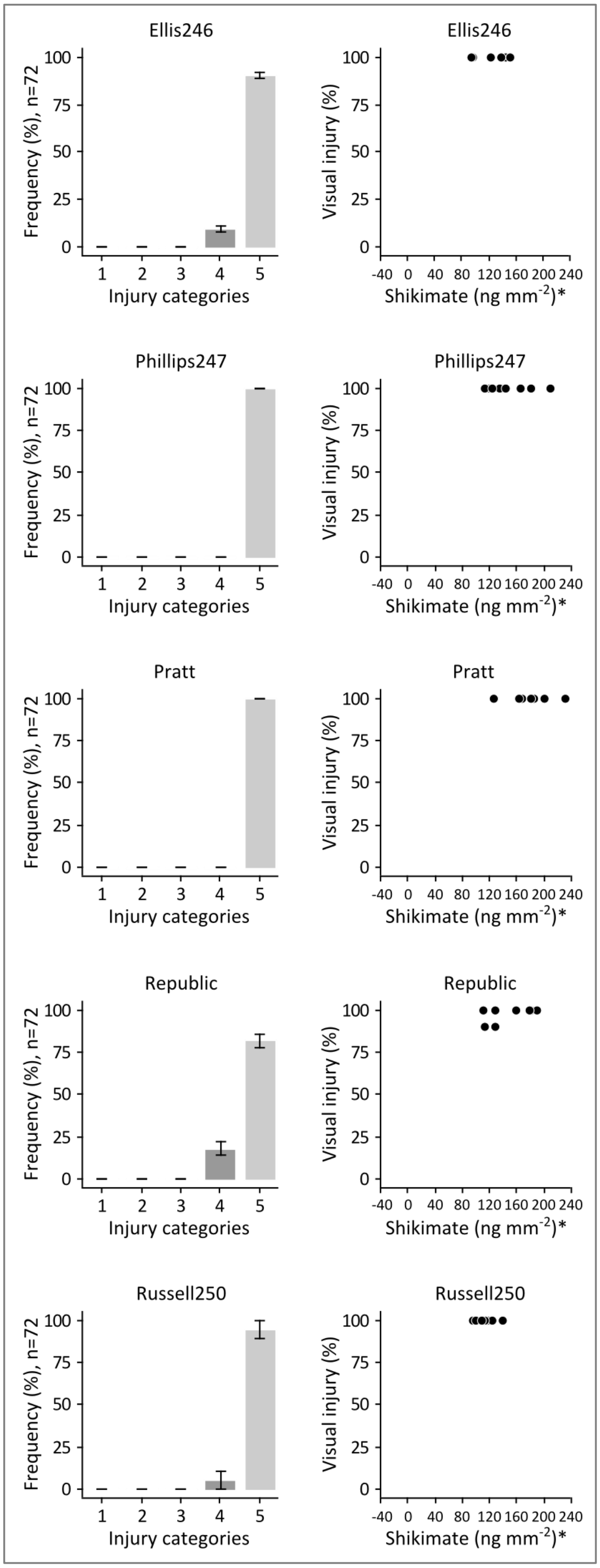

Ellis246
Frequency (%), n=72
Injury categories
1 2 3 4 5
0 25 50 75 100
Ellis246
Visual injury (%)
Shikimate (ng mm^{-2})*
-40 0 40 80 120 160 200 240
Phillips247
Frequency (%), n=72
Injury categories
Phillips247
Visual injury (%)
Shikimate (ng mm^{-2})*
Pratt
Frequency (%), n=72
Injury categories
Pratt
Visual injury (%)
Shikimate (ng mm^{-2})*
Republic
Frequency (%), n=72
Injury categories
Republic
Visual injury (%)
Shikimate (ng mm^{-2})*
Russell250
Frequency (%), n=72
Injury categories
Russell250
Visual injury (%)
Shikimate (ng mm^{-2})*

Figure 3.4 Injury ratings and shikimate accumulation results for category A kochia populations. General description of the category and geographic location of the populations are shown in Table 3.1. Kochia plants (10- to 12-cm tall) were treated with 0.84 kg ae ha^{-1} glyphosate with 2% w/v AMS (n=72) and rated for injury 18 days after treatment. Injury categories 1, 2, 3, 4, and 5 on X-axis represent 0-30, 31-60, 61-90, 91-99, and 100% injury, respectively. For shikimate accumulation assay, 5-mm leaf discs were treated in 100 µM glyphosate solution and were incubated under contiguous light for 16 h (n=9).

*shikimate accumulation (ng mm^{-2} leaf disc), shikimate accumulation in control discs subtracted

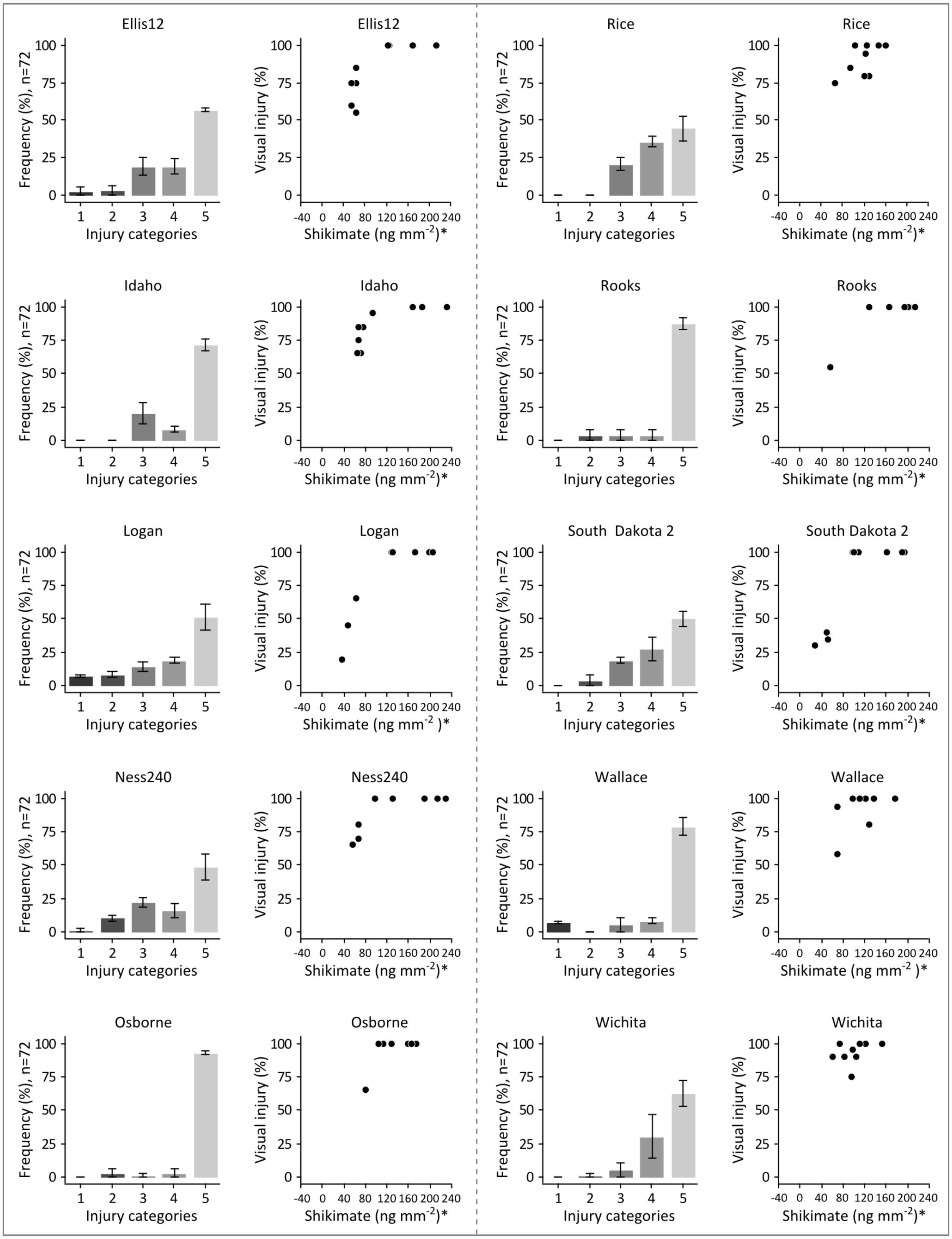

Ellis12
Frequency (%), n=72
Injury categories
Ellis12
Visual injury (%)
Shikimate (ng mm^{-2})*
Rice
Frequency (%), n=72
Injury categories
Rice
Visual injury (%)
Shikimate (ng mm^{-2})*
Idaho
Frequency (%), n=72
Injury categories
Idaho
Visual injury (%)
Shikimate (ng mm^{-2})*
Rooks
Frequency (%), n=72
Injury categories
Rooks
Visual injury (%)
Shikimate (ng mm^{-2})*
Logan
Frequency (%), n=72
Injury categories
Logan
Visual injury (%)
Shikimate (ng mm^{-2})*
South Dakota 2
Frequency (%), n=72
Injury categories
South Dakota 2
Visual injury (%)
Shikimate (ng mm^{-2})*
Ness240
Frequency (%), n=72
Injury categories
Ness240
Visual injury (%)
Shikimate (ng mm^{-2})*
Wallace
Frequency (%), n=72
Injury categories
Wallace
Visual injury (%)
Shikimate (ng mm^{-2})*
Osborne
Frequency (%), n=72
Injury categories
Osborne
Visual injury (%)
Shikimate (ng mm^{-2})*
Wichita
Frequency (%), n=72
Injury categories
Wichita
Visual injury (%)
Shikimate (ng mm^{-2})*
0
25
50
75
100
1
2
3
4
5
-40
0
40
80
120
160
200
240

Figure 3.5 Injury ratings and shikimate accumulation results for category B kochia populations. General description of the category and geographic location of the populations are shown in Table 3.1. Kochia plants (10- to12-cm tall) were treated with 0.84 kg ae ha^{-1} glyphosate with 2% w/v AMS (n=72) and rated for injury 18 days after treatment. Injury categories 1, 2, 3, 4, and 5 on X-axis represent 0-30, 31-60, 61-90, 91-99, and 100% injury, respectively. For shikimate accumulation assay, 5-mm leaf discs were treated in 100 µM glyphosate solution and were incubated under contiguous light for 16 h (n=9).

*shikimate accumulation (ng mm^{-2} leaf disc), shikimate accumulation in control discs subtracted

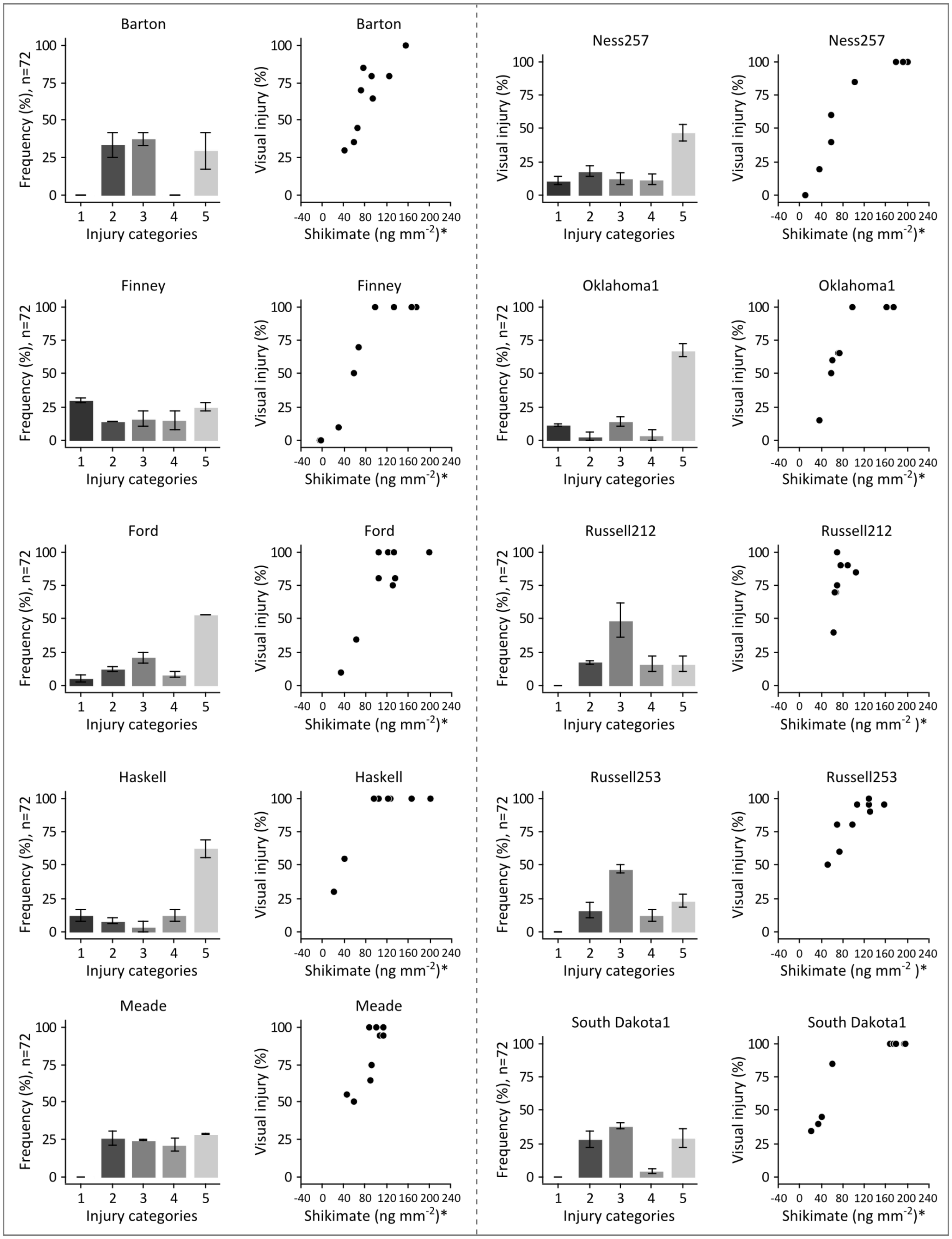

Barton
Frequency (%), n=72
Injury categories
Barton
Visual injury (%)
Shikimate (ng mm^{-2})*
Ness257
Visual injury (%)
Injury categories
Ness257
Visual injury (%)
Shikimate (ng mm^{-2})*
Finney
Frequency (%), n=72
Injury categories
Finney
Visual injury (%)
Shikimate (ng mm^{-2})*
Oklahoma1
Frequency (%), n=72
Injury categories
Oklahoma1
Visual injury (%)
Shikimate (ng mm^{-2})*
Ford
Frequency (%), n=72
Injury categories
Ford
Visual injury (%)
Shikimate (ng mm^{-2})*
Russell212
Frequency (%), n=72
Injury categories
Russell212
Visual injury (%)
Shikimate (ng mm^{-2})*
Haskell
Frequency (%), n=72
Injury categories
Haskell
Visual injury (%)
Shikimate (ng mm^{-2})*
Russell253
Frequency (%), n=72
Injury categories
Russell253
Visual injury (%)
Shikimate (ng mm^{-2})*
Meade
Frequency (%), n=72
Injury categories
Meade
Visual injury (%)
Shikimate (ng mm^{-2})*
South Dakota1
Frequency (%), n=72
Injury categories
South Dakota1
Visual injury (%)
Shikimate (ng mm^{-2})*
0
25
50
75
100
1
2
3
4
5
-40
0
40
80
120
160
200
240

Figure 3.6 Injury ratings and shikimate accumulation results for category C kochia populations. General description of the category and geographic location of the populations are shown in Table 3.1. Kochia plants (10- to 12-cm tall) were treated with 0.84 kg ae ha^{-1} glyphosate with 2% w/v AMS (n=72) and rated for injury 18 days after treatment. Injury categories 1, 2, 3, 4, and 5 on X-axis represent 0-30, 31-60, 61-90, 91-99, and 100% injury, respectively. For shikimate accumulation assay, 5-mm leaf discs were treated in 100 µM glyphosate solution and were incubated under contiguous light for 16 h (n=9).

*shikimate accumulation (ng mm^{-2} leaf disc), shikimate accumulation in control discs subtracted

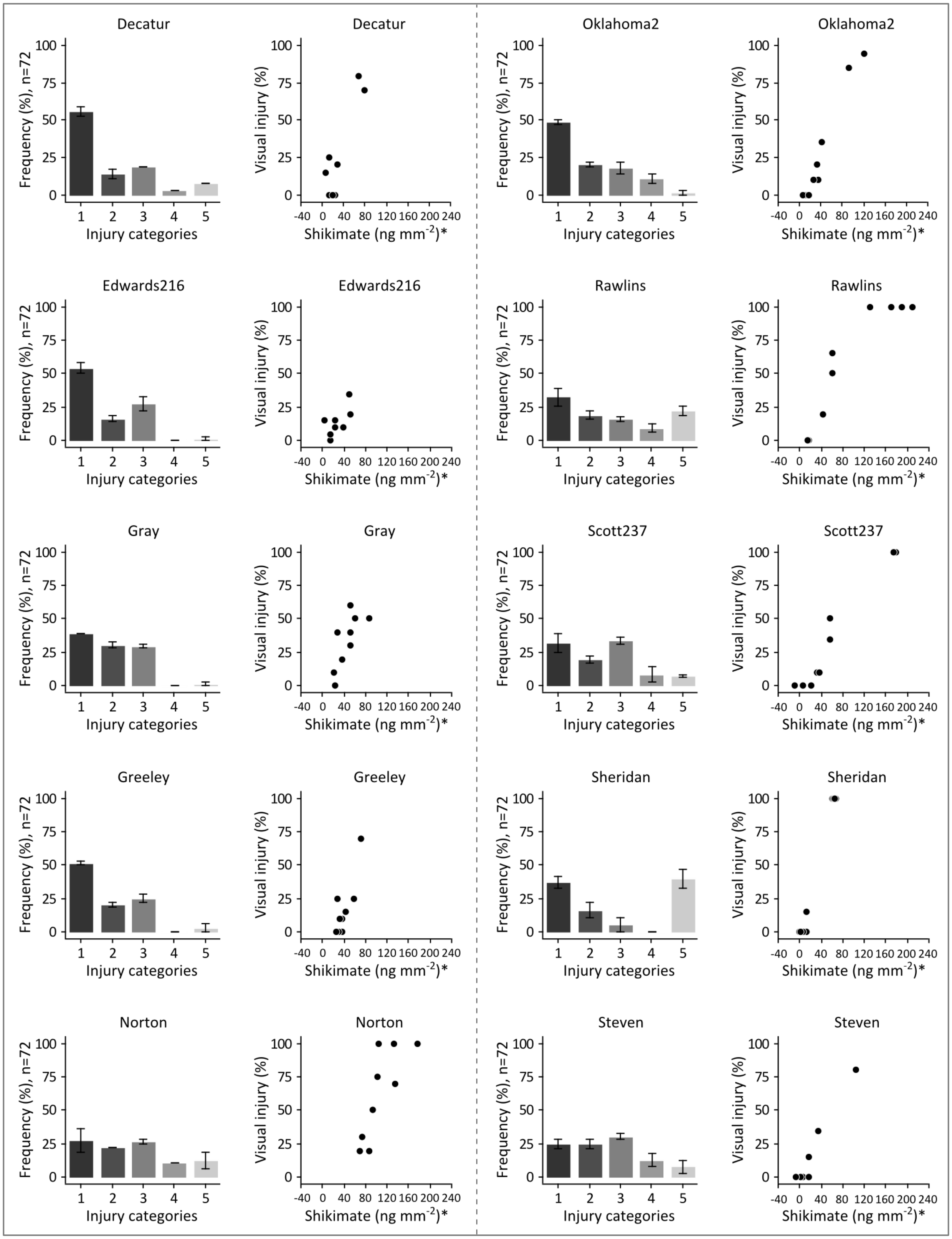

Decatur
Decatur
Oklahoma2
Oklahoma2
Edwards216
Edwards216
Rawlins
Rawlins
Gray
Gray
Scott237
Scott237
Greeley
Greeley
Sheridan
Sheridan
Norton
Norton
Steven
Steven
Frequency (%), n=72
Visual injury (%)
Injury categories
Shikimate (ng mm^{-2})*
0
25
50
75
100
1
2
3
4
5
-40
0
40
80
120
160
200
240

Figure 3.7 Injury ratings and shikimate accumulation results for category D kochia populations. General description of the category and geographic location of the populations are shown in Table 3.1. Kochia plants (10- to 12-cm tall) were treated with 0.84 kg ae ha^{-1} glyphosate with 2% w/v AMS (n=72) and rated for injury 18 days after treatment. Injury categories 1, 2, 3, 4, and 5 on X-axis represent 0-30, 31-60, 61-90, 91-99, and 100% injury, respectively. For shikimate accumulation assay, 5-mm leaf discs were treated in 100 µM glyphosate solution and were incubated under contiguous light for 16 h (n=9).

*shikimate accumulation (ng mm^{-2} leaf disc), shikimate accumulation in control discs subtracted

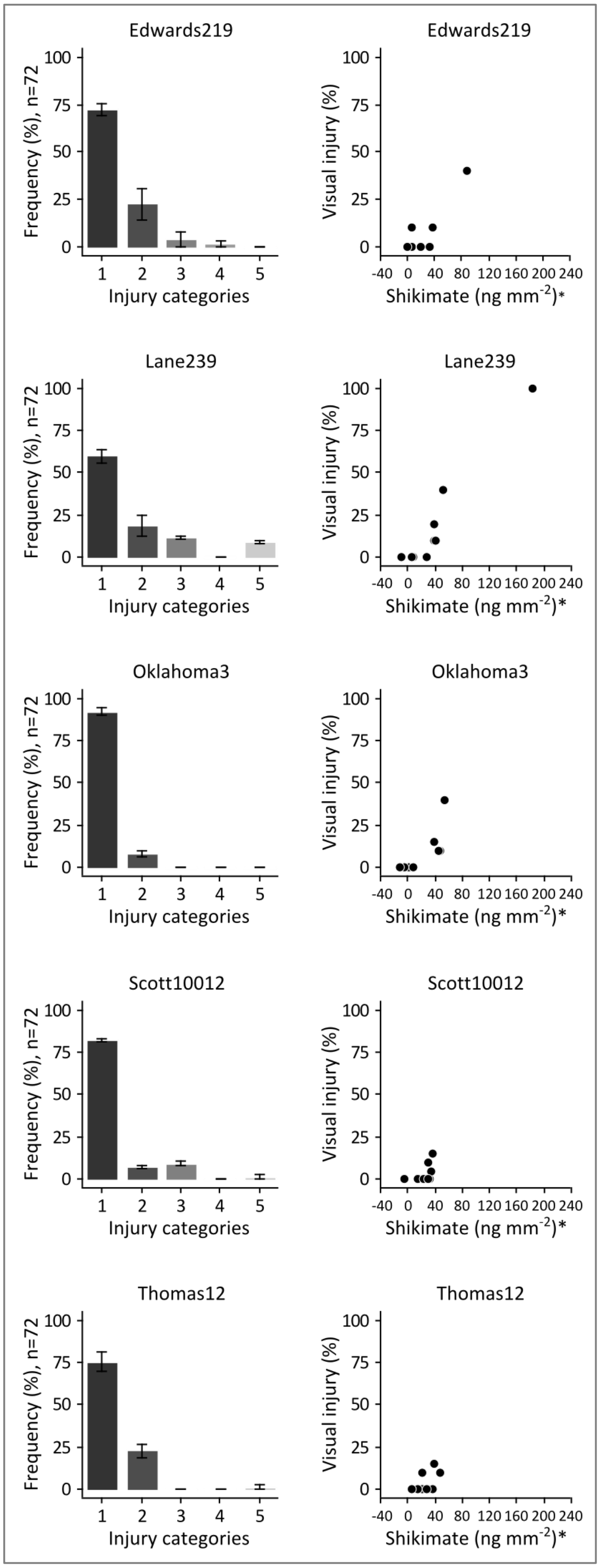

Edwards219
Frequency (%), n=72
Injury categories
Visual injury (%)
Shikimate (ng mm^{-2})*
Lane239
Oklahoma3
Scott10012
Thomas12
0
25
50
75
100
1
2
3
4
5
-40
0
40
80
120
160
200
240

Figure 3.8 Injury ratings and shikimate accumulation results for category E kochia populations. General description of the category and geographic location of the populations are shown in Table 3.1. Kochia plants (10- to 12-cm tall) were treated with 0.84 kg ae ha^{-1} glyphosate with 2% w/v AMS (n=72) and rated for injury 18 days after treatment. Injury categories 1, 2, 3, 4, and 5 on X-axis represent 0-30, 31-60, 61-90, 91-99, and 100% injury, respectively. For shikimate accumulation assay, 5-mm leaf discs were treated in 100 µM glyphosate solution and were incubated under contiguous light for 16 h (n=9).

*shikimate accumulation (ng mm^{-2} leaf disc), shikimate accumulation in control discs subtracted

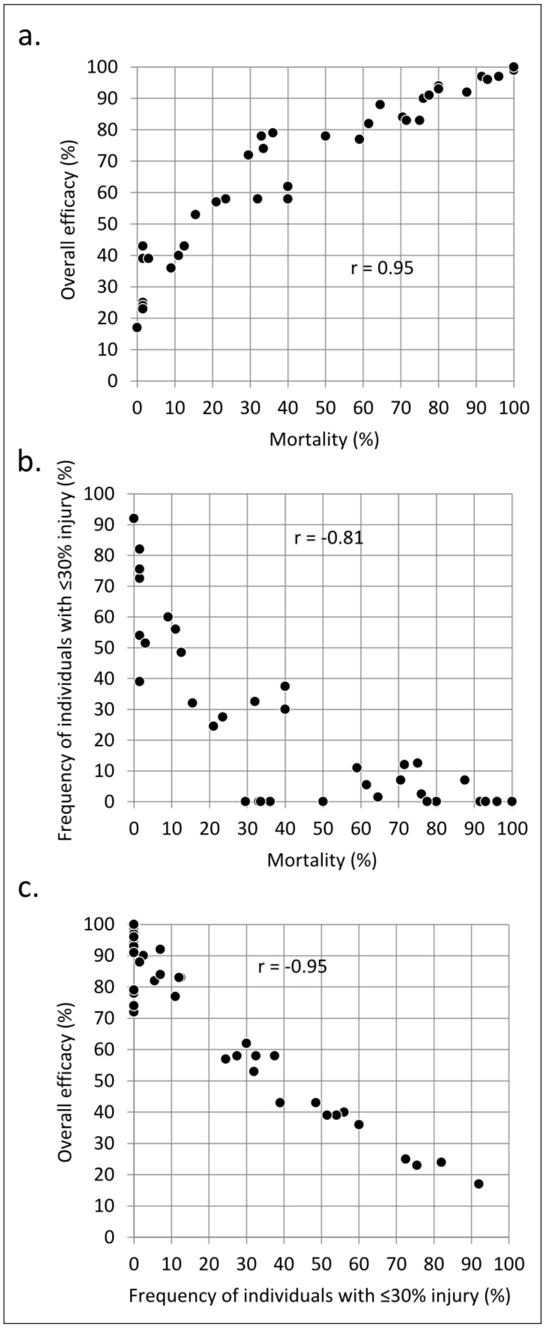


Figure 3.9 Relationships among percent mortality, percent efficacy, and frequency of individuals with ≤30% injury assessed 18 days after 0.84 kg ae ha^{-1} glyphosate treatment. (a) Relationship between mortality and overall efficacy, (b) relationship between mortality and frequency of individuals with ≤30% injury, and (c) relationship between frequency of individuals with overall efficacy.

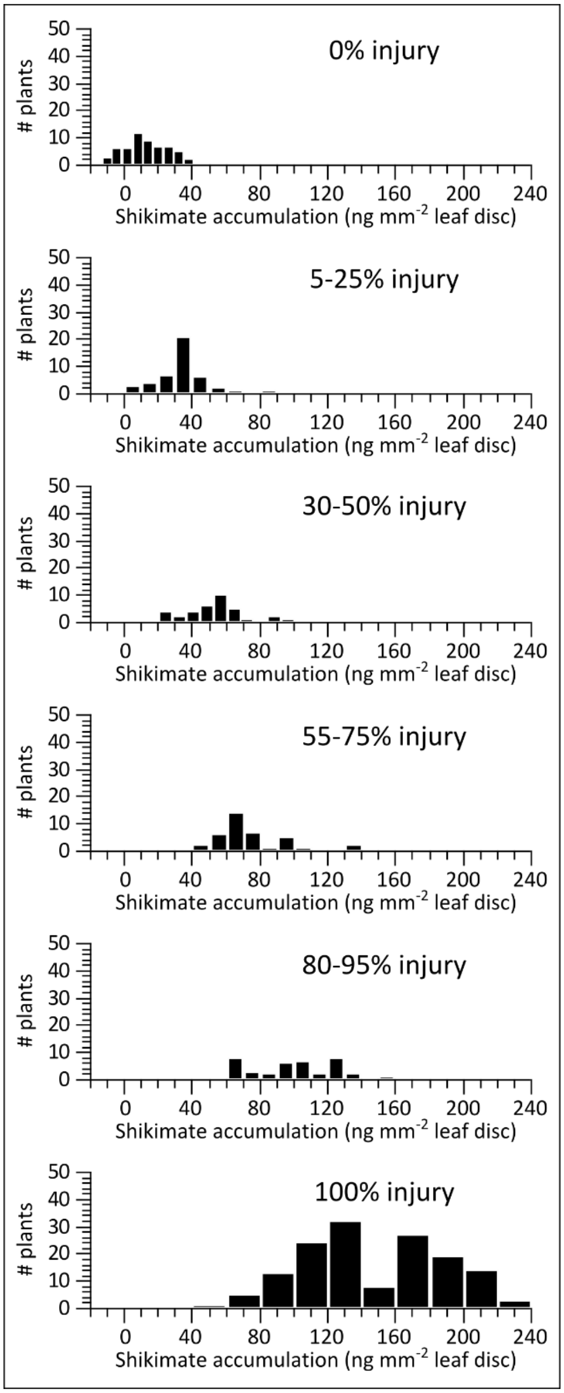


Figure 3.10 Shikimate accumulation in kochia plants grouped by injury levels 18 days after 0.84 kg ae ha^{-1} glyphosate treatment. Shikimate accumulation was measured by treating 5-mm leaf discs in 100 µM glyphosate solution and incubating under contiguous light for 16 h.

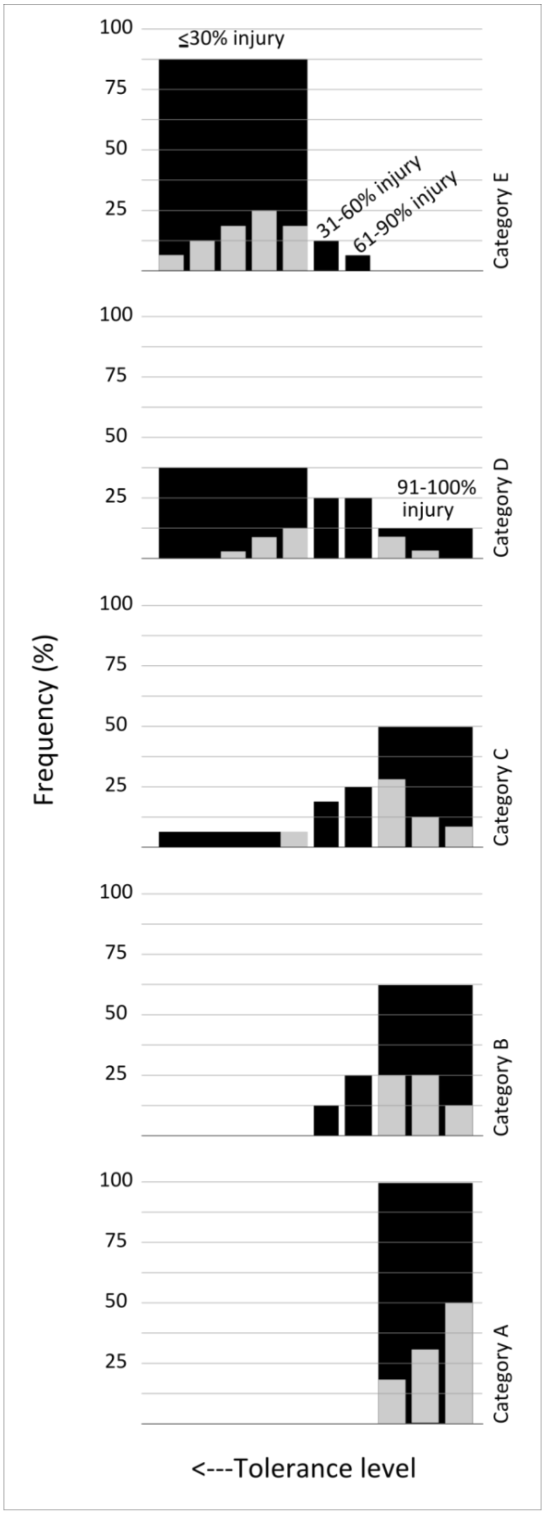


Figure 3.11 Illustrative population composition of five kochia population categories. Vertical dark-colored bars represent ≤30, 31-60, 61-90, and 91-100% injury (with 0.84 ae kg ae ha^{-1}

glyphosate) from left to right, respectively. Frequency of these injury categories reflects average frequency of corresponding injury categories shown in Figures 3.4, 3.5, 3.6, 3.7, and 3.8 for each population category. Light-colored bars within the dark-colored bars represent predicted frequencies based on results from Table 3.2.

Chapter 4 - Absorption and Translocation of ^{14}C-glyphosate in Glyphosate-susceptible and Glyphosate-resistant Kochia Populations

Abstract

Independent studies have reported amplification of the target site and no role of absorption and translocation of glyphosate in glyphosate-resistant (GR) kochia populations. The objectives of this study were to investigate if altered absorption and/or translocation of glyphosate contributed to resistance in a GR kochia population that was found to have amplified EPSPS gene copies. Glyphosate-resistant (KS-R5) and glyphosate-susceptible (Ellis) kochia populations were treated with ^{14}C-glyphosate, and recovered ^{14}C-glyphosate in wash solution from treated leaves and dissected plant parts was measured at 24, 48, 72, and 96 h after treatment. Absorption of ^{14}C-glyphosate was higher in the Ellis population (63 to 66%) than in the KS-R5 population at all harvest times; however, differences were usually less than 10% of the total applied. Translocation to plant parts above- and below-treated leaves was similar (<5%) in both populations. More than 80% of the recovered ^{14}C-glyphosate in plants remained in the treated leaves of both populations at all harvest times. The KS-R5 population translocated more ^{14}C-glyphosate (14%) to roots than the Ellis population (7%), but only at 96 h after treatment. Nominal differences in absorption and translocation of glyphosate likely do not contribute to the differential response of these populations to glyphosate observed in field studies.

Introduction

Glyphosate-resistant (GR) kochia is widespread in western Kansas, and recently its presence has also been reported in other Great Plains states and southern Canada (Chapter 1, Heap 2013). Under repeated selection pressure, weed populations can evolve to resist normal use rates of glyphosate by several mechanisms (Perez-Jones and Mallory-Smith 2010). Wiersma (2012) investigated target site mechanisms in northern Great Plains kochia populations and suggested EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene amplification may be the mechanism of glyphosate resistance. Waite et al. (2013) observed similar absorption and translocation of ^{14}C-glyphosate in western Kansas kochia populations that showed varied tolerance to glyphosate. However, glyphosate absorption and translocation, and target site mechanisms have not been studied in the same kochia populations.

Both target site and non-target site mechanisms have been reported to confer resistance to glyphosate in some weed species. Reduced absorption and increased vacuolar sequestration of glyphosate were reported in GR Italian ryegrass (Michitte et al. 2007) and horseweed (Ge et al. 2009). Feng et al. (2004), and Koger and Reddy (2005) reported reduced glyphosate translocation to young leaves of GR horseweed populations. This mechanism has also been observed in Italian ryegrass (Michitte et al. 2007) and rigid ryegrass (Wakelin et al. 2004, Yu et al. 2009). While altered absorption and translocation is the most commonly reported mechanism of glyphosate resistance in weed species, target site mutation and increased expression of the target site has been found in some species. Baerson et al. (2002), Jasieniuk et al. (2008), and Wakelin and Preston (2006) reported mutation in glyphosate target site gene, EPSPS, at PRO-106 in GR goosegrass, Italian ryegrass and rigid ryegrass, respectively. Other than in kochia, amplified copies of EPSPS gene has been reported as the primary mechanism of glyphosate resistance in Palmer amaranth (Gaines et al. 2010) and rigid ryegrass (Salas et al. 2012). Thus, it is important to investigate if multiple mechanisms of resistance exist in GR weed populations.

Wiersma (2012) found increased EPSPS expression in kochia plants that had amplified copies of the EPSPS gene. Results indicated a strong relationship between EPSPS gene copy number and level of glyphosate resistance. Since multiple mechanisms of evolved herbicide resistance have been documented in weed species, non-target site mechanisms including altered absorption and/or translocation may contribute to glyphosate resistance in kochia. This study was conducted to determine if altered absorption and/or translocation contributed to glyphosate resistance in a kochia population that was found to have amplified copies of the EPSPS gene.

Materials and Methods

Plant Materials and Growth Conditions

Confirmed GR (Wiersma 2012) and known GS (Chapter 1) kochia populations were chosen for this study. Seeds of GR (identified as KS-R5) and GS (identified as Ellis) populations were collected in fall 2010 from Scott County and Ellis County, KS, respectively. Kochia seeds were planted separately in plastic trays with commercial potting mix in the greenhouse. Plants were grown under 25/20 (±2) C day/night with 15/9 h day/night photoperiod, supplemented with 200 µmol $m^{-2}s^{-1}$ illumination provided with sodium vapor lamps. Following emergence, individual seedlings at the two-leaf stage (approx. 2 cm tall) were removed from the trays with most roots intact. Roots were cleaned by dipping in water. The seedlings were then transplanted to plastic 4-cm-diam. plastic pots filled with Turface (Turface MVP®, PROFILE Products LLC, Buffalo Grove, IL). Plants were grown in Turface for easier root extraction. The Turface was soaked in water for 10 to15 min before filling pots. After transplanting, the pots were covered with clear plastic propagation domes for two weeks to induce root establishment. Plants were watered as needed and fertilized (Miracle-Gro, Scotts Miracle-Gro Products Inc., Marysville, OH) with a solution containing 1.2 g L^{-1} total nitrogen, 0.4 g L^{-1} phosphorus, and 0.8 g L^{-1} potassium once seven days after transplanting. No significant growth was visible for 10 days after transplanting. The domes were removed when the plants were 2 to 3 cm tall.

^{14}C-glyphosate Application

Plants were 5 to 6 cm tall with 6 to 8 leaves at the time of ^{14}C-glyphosate application. The first two fully-expanded leaves from the top of plants were marked and covered with aluminum foil. Glyphosate (Roundup Weathermax®, Monsanto Company, St. Louis MO) at 0.84 k g ae ha^{-1} was applied to the kochia plants. Glyphosate was applied with a moving single-nozzle bench-type sprayer (Research Track Sprayer, De Vries Manufacturing, Hollandale, MN) equipped with a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., Wheaton, IL) delivering 168 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. Glyphosate treatment included ammonium sulfate (AMS 2% w/v). Spray droplets were allowed to dry (approx. 30 min) and the aluminium foil was removed from the leaves. Two drops of 2.2 µL glyphosate solution (26.6 mM = 0.84 kg ae ha^{-1} @ 187 L ha^{-1} carrier volume) containing 0.33 kBq μL^{-1} of ^{14}C-glyphosate and unlabeled glyphosate (Roundup Weathermax, Monsanto Company, St. Louis MO) was applied with a microsyringe to the middle of the adaxial surface (approx. 1 cm apart) of the marked leaves (identified hereafter as treated leaves) avoiding midrib.

Measurement of Absorption and Translocation

Plant parts (treated leaves, above-treated part, below-treated part, and roots) were harvested separately at 24, 48, 72, and 96 h after treatment. The treated leaves were immediately rinsed twice for 30 s with 5 ml of a wash solution containing 10% v/v ethanol and 0.5% v/v Tween 20 (Thermo Fisher Scientific Inc) in a 20-ml scintillation vial. Then 10 ml of scintillation cocktail (Ecolite (+), Thermo Fisher Scientific Inc.) was added to vials for measuring unabsorbed radioactivity. All plant parts including the washed treated leaves were dried for 72 h at 65 C and stored at 40 C until oxidized. Individual plant parts were oxidized using a biological oxidizer (OX-510; R.J. Harvey Instrument Co, Hillsdale, NJ) and evolved CO_2 was trapped in 15 ml of scintillation cocktail. Radioactivity recovered from each plant, including the rinsate, was determined using liquid scintillation spectrometry (Tricarb 2100 TR Liquid Scintillation Analyzer; Packard Instrument Co., Meriden, CT).

Herbicide absorption was expressed as percentage of total radioactivity applied (Equation 4.1).

$$Herbicide\ absorption(\%) = \frac{Radioactivity\ applied - Radioactivity\ in\ rinsate}{Radioacivity\ applied} \qquad \text{Eq. 4.1}$$

Herbicide distribution (translocation) within the plant was determined by expressing radioactivity recovered in a given plant part as a percentage of the total radioactivity recovered (Equation 4.2).

$$Herbicide\ in\ a\ given\ plant\ part\ (\%) = \frac{Radioactivity\ recovered\ in\ a\ given\ plant\ part}{Total\ radioctivity\ recovered\ in\ plant} \quad \text{Eq. 4.2}$$

Experimental Design and Statistical Analysis

The experiment was a completely randomized design with factorial arrangements of kochia populations, harvest times and plant parts. The treatments were replicated four times and the experiment was repeated. Data were analyzed only for kochia populations for individual plant parts or absorption separately for each harvesting time. Means were compared using two-tailed t-test at $\alpha = 0.05$.

Results

Only 65 to 75% of the applied ^{14}C-glyphosate was recovered from both resistant and susceptible populations at all harvest timings. Absorption of ^{14}C-glyphosate was higher in the Ellis than in the KS-R5 population at all harvest intervals (Table 4.1). Absorption in Ellis and KS-R5 populations ranged from 63 to 66% and 54 to 59% of total applied radioactivity, respectively.

The percentage of recovered radioactivity in different plant parts (Equation 4.2) of glyphosate-resistant and -susceptible kochia biotypes is shown in Table 4.1. At all harvest times, most of the recovered radioactivity remained in the treated leaves of both populations. Percent radioactivity in the treated leaves was about 10% higher at 24 and 48 h (90 to 93%) compared to 72 and 96 h (80 to 84%).

Less than 5% of applied radioactivity was recovered from plant parts above-treated leaves and below-treated leaves, and the amount was similar for both populations at all harvest times. Recovered amounts of radioactivity were 0.3 to 0.4% in above-treated leaves at 24 h and gradually increased to 3.2 to 3.4 at 96 h. In below-treated plant parts, recovered radioactivity generally remained similar across harvest times and ranged from 2.1 to 5%.

The amount of radioactivity in roots was similar between populations at all harvest timings up to 72 h. Three to six percent of the radioactivity was recovered from roots at 24 and 48 h, however, the amount increased to 10-12% by 72 h. Thereafter, at 96 h, the amount decreased to 7.9% in the Ellis population. In contrast, 14% of the radioactivity was recovered from roots of the KS-R5 population at 96 h.

Discussion

More than 50% of applied ^{14}C-glyphosate was absorbed by 24 h in both populations and the absorption thereafter remained similar. Kniss et al. (2011) suggested non-linear regression models to estimate A_{max}, the time at which maximum absorption occurs. In this study, maximum absorption occurred sometime before 24 h in both populations; hence, it was not possible to accurately measure the differences in rate of absorption between the populations. Absorption was higher in the Ellis population than in the KS-R5 population at all harvest times; however, differences usually were less than 10% of the total applied. Reduced absorption was observed in glyphosate-resistant Italian ryegrass (Michitte et al. 2007), but the resistant population absorbed 40% less glyphosate compared to the susceptible population. In addition, spray retention also was 35% less in resistant than in the susceptible Italian ryegrass population.

At least 80% of the recovered radioactivity remained in the treated leaves until 96 h and the amount was similar in both populations. However, differences in the amount of radioactivity recovered in treated leaves have been shown in glyphosate-resistant and -susceptible populations of some weed species. Perez-Jones et al. (2007) and Koger and Reddy (2005), reported greater glyphosate retention in the treated leaves of glyphosate-resistant than in glyphosate-susceptible Italian ryegrass and horseweed populations, respectively. Considering the amount not absorbed and not recovered in kochia populations, the amount that remained in the treated leaves is only about one-third of the total radioactivity applied.

Similarly, no differences were observed in the amount of radioactivity translocated to above-treated and below-treated plant parts. However, difference in amounts recovered in roots was evident between the populations at 96 h. This is likely because of the discrepancy in rates of physiological processes between the populations as the plants from Ellis population were severely injured while the plants from KSR-5 showed no or minimal injury at 96 h.

The nominal differences in absorption and translocation between these two populations would not significantly contribute to tolerance/susceptibility to glyphosate. Such minor differences may be due to natural variations even among glyphosate-resistant kochia populations. Moreover, a bias created due to overspraying of unlabeled glyphosate on these populations may have affected absorption and translocation of ^{14}C-glyphosate (Dill et al. 2010). Glyphosate absorption and translocation results were similar to other kochia populations that differed in tolerance to glyphosate (Waite et al. 2013). In addition, Culpepper et al. (2006) reported similar translocation of glyphosate in glyphosate-resistant and-susceptible Palmer amaranth. As most of the absorption occurred within 24 h of glyphosate treatment, earlier sampling times closer to application time would better describe the rate of absorption. Nonetheless, differences in initial absorption rates may not result in large variance in tolerance. While other non-target mechanisms may partially contribute to the glyphosate resistance in kochia, these results seemingly eliminate glyphosate absorption and translocation as possibilities.

References

Baerson, S. R., D. J. Rodriguez, M. Tran, Y. Feng, N. A. Best, and G. M. Dill. 2002. Glyphosate-resistant goosegrass: identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. Plant Physiol. 129:1265-1275.

Culpepper, S., T. L. Grey, W. K. Vencil, J. M. Kichler, T. M. Webster, S. M. Brown, A. C. York, J. W. Davis, and W. W. Hanna. 2006. Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) confirmed in Georgia. Weed Sci. 54:620-626.

Dill, G. M., R. D. Sammons, P. C. C. Feng, F. Kohn, K. Kretzmer, A. Mehrsheikh, M. Bleeke, J. L. Honegger, D. Farmer, D. Wright, and E. A. Haupfear. 2010. Glyphosate: discovery, developments, applications, and properties. In: Glyphosate resistance in crops and weeds: History, development, and management. Ed Nandula, V. K. John Wiley & Sons, Inc. pp 199-148.

Feng, P.C.C., M. Tran, T. Chiu, R. D. Sammons, G. R. Heck, and C. A. Jacob. 2004. Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): retention, uptake, translocation, and metabolism. Weed Sci. 52:498-505.

Gaines, T. A., W. Zhang, D. Wang, B. Bukun, S. T. Chisholm, D. L. Shaner, S. J. Nissen, W. L. Patzoldt, P. J. Tranel, A. S. Culpepper, T. L. Grey, T. M. Webster, W. K. Vencill, R. D. Sammons, J. Jiang, C. Preston, J. E. Leach, and P. Westra. 2010. Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. PNAS. 107:1029-1034.

Ge, X., D. A. d'Avignon, J. J. H. Ackerman, and R. D. Sammons. 2009. Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism. Pest Manag. Sci. 66:345-348.

Heap, I. The International Survey of Herbicide Resistant Weeds. Online. Internet. Accessed June 19, 2013. Available www.weedscience.com

Jasieniuk, M., R. Ahmad, A. M. Sherwood, J. L. Firestone, A. Perez-Jones, W. T. Lanini, C. Mallory-Smith, and Z. Stednick. 2008. Glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in California: distribution, response to glyphosate, and molecular evidence for an altered target enzyme. Weed Sci. 56:496-502.

Kniss, A. R., J. D. Vassios, S. J. Nissen, and C. Ritz. 2011. Nonlinear regression analysis of herbicide absorption studies. Weed Sci. 59:601-610.

Koger, C. H. and K. N. Reddy. 2005. Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). Weed Sci. 53:84-89.

Michitte, P., R. De Prado, N. Espinosa, J. P. Ruiz-Santaella, and C. Gauvrit. 2007. Mechanisms of resistance to glyphosate in a ryegrass (*Lolium multiflorum*) biotype from Chile. Weed Sci. 55:435-440.

Perez-Jones, A., K. W. Park, N. Polge, J. Colquhoun, and C. A. Mallory-Smith. 2007. Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. Planta 226:395-404.

Perez-Jones, A. and C. Mallory-Smith. 2010. Biochemical mechanisms and molecular basis of evolved glyphosate resistance in weeds species. In: Glyphosate resistance in crops and weeds: History, development, and management. Ed Nandula, V. K. John Wiley & Sons, Inc. pp 199-148.

Salas, R. A., F. E. Dayan, Z. Pan, S. B. Watson, J. W. Dickson, R. C. Scott, and N. R. Burgos. 2012. EPSPS gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) from Arkansas. Pest Manag. Sci. 68:1223-1230.

Waite, J., C. R. Thompson, D. E. Peterson, R. S. Currie, B. L. S. Olson, P. W. Stahlman, and K. Al-Khatib. 2013. Differential kochia (*Kochia scoparia*) populations response to glyphosate. Weed Sci. 61:193-200.

Wakelin, A. M. and C. Preston. 2006. A target-site mutation is present in a glyphosate-resistant *Lolium rigidum* population. Weed Res. 46:432-440.

Wakelin, A. M., D. F. Lorraine-Colwill, and C. Preston. 2004. Glyphosate resistance in four different populations of *Lolium rigidum* as associated with reduced translocation of glyphosate to meristematic zones. Weed Res. 44: 453-459.

Wiersma, A. 2012. Regional whole plant and molecular response of *Kochia scoparia* to glyphosate. M.S. thesis. Colorado State University. Fort Collins, Co. 63 p.

Table 4.1 Absorption and translocation of ^{14}C-glyphosate in glyphosate-resistant and glyphosate-susceptible kochia populations.

	24 h	48 h	72 h	96 h
	------------------------------ % of applied[a]			------------------------------
Absorption				
Ellis	65	63	65	66
KS-R5	54 *	54 *	59 *	57 *
	------------------------------ % of recovered[b]			------------------------------
Treated leaves				
Ellis	93	93	82	84
KS-R5	92	90	83	80
Above-treated leaves				
Ellis	0.3	0.4	2.5	3.2
KS-R5	0.4	0.3	3.0	3.4
Below-treated leaves				
Ellis	2.1	3.1	3.2	4.6
KS-R5	2.1	5.0	3.7	2.1
Roots				
Ellis	4.2	3.1	12	7.9
KS-R5	5.8	4.2	10	14 *

Values are the mean of eight replications. Asterisks indicate the mean value differs significantly from the value for the Ellis population at $\alpha = 0.05$ and the absence of asterisks indicate mean values within plant parts do not differ significantly

[a] equation 4.1

[b] equation 4.2

Chapter 5 - Shikimate Accumulation in Glyphosate-treated Leaf Discs in Relation to EPSPS Gene Copy Number in Kochia

Abstract

Levels of resistance to glyphosate vary among and within glyphosate-resistant (GR) kochia populations. The objectives of this study were to (1) determine the amount of shikimate accumulation in leaf discs treated with a series of glyphosate concentrations at different incubation times, and (2) evaluate the relationship between shikimate accumulation and EPSPS gene copy number in GR and glyphosate-susceptible (GS) kochia plants. Leaf discs of kochia plants (8 GR plants and 3 GS plants) were treated with 0 to 800 µM glyphosate solution and incubated for 16 h or were treated with 100 µM of glyphosate and incubated for 12, 24, and 48 h. Relative EPSPS:ALS gene copy numbers in the kochia plants were determined using qPCR. Generally, the amount of shikimate accumulation was lower in the leaf discs of GR plants than in the leaf discs of GS plants across all glyphosate concentrations. Similarly, leaf discs of GR plants accumulated less shikimate at all incubation times. In both GR and GS kochia plants, the amount of shikimate accumulated in leaf discs was much greater with longer incubation times than with higher concentrations of glyphosate. Levels of shikimate accumulation in leaf discs correlated well with the EPSPS gene copy across all glyphosate concentrations and incubation times (R^2 = 0.52 to 0.88). Plants with more copies of EPSPS gene, generally, accumulated less shikimate.

Introduction

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC25.5.1.19) (Steinrucken and Amrbein 1980). EPSPS is one of the key enzymes in the shikimate pathway (Herrmann and Weaver 1999) and catalyzes transfer of the enolpyruvyl moiety from phospoenolpyruvate (PEP) to shikimate-3-phosphate (S3P) to produce 5-enolpyruvylshikimate-3-phosphate (EPSP). As glyphosate is an analog to PEP, glyphosate inhibits EPSPS by binding to EPSPS-S3P binary complex forming an EPSPS-S3P-glyphosate ternary complex (Alibhai and Stalling 2001). Thus, presence of glyphosate molecules in the plant system inhibits the shikimic acid (shikimate) pathway and, thereby results in rapid accumulation of shikimate.

Glyphosate-resistant (GR) crops and GR weed biotypes avoid or lessen EPSPS inhibition by glyphosate through several mechanisms. Documented mechanisms of glyphosate resistance in weed biotypes include mutation in the target site (EPSPS) gene (Devine and Shukla 2000, Preston and Mallory-Smith 2001), amplification (Gaines et al. 2010, Salas et al. 2012, Wiersma 2012) and overexpression (Pline-Srnic 2006) of the target site gene, and altered uptake and translocation of glyphosate (Koger and Reddy 2005, Perez-Jones et al. 2007, Preston 2004). Engineered GR crops have a glyphosate-insensitive form of EPSPS, *epsps CP4* gene, together with glyphosate oxidoreductase (GOX) gene for glyphosate metabolism in some crops (Funke et al. 2006, Green 2009). The target site amplification has been suggested to be the mechanism of glyphosate resistance in GR kochia [K*ochia scoparia* (L.) Schrad.] (Wiersma 2012). The amplification of the EPSPS gene in GR kochia results in increased expression of EPSPS, thus preventing complete inhibition of the shikimate pathway when sprayed with normal use rate of glyphosate.

Shikimate accumulation assay is used in glyphosate resistance and crop injury studies (Koger et al. 2005a, Koger et al. 2005b, Gaines et al. 2012, Henry et al. 2007, Mueller et al. 2003, Simarmata et al. 2003). Shikimate accumulation assays using methodology described by Shaner et al. (2005) were used in glyphosate resistance studies in kochia populations (Wiersma 2012, Chapter 1 and 3). Both studies used a single concentration of glyphosate. The amount of shikimate accumulated was usually more in susceptible than in resistant kochia plants as reported in other weed species (Muller et al. 2008).

Glyphosate resistance levels in individual plants vary widely among GR kochia populations and the level of resistance appears to be strongly related to EPSPS gene copies in the plants (Wiersma 2012). In a very short period after the first confirmation GR kochia in Kansas, the GR kochia populations were estimated to be present in more than one-third of croplands in western Kansas (Chapter 1 and 2), and now, its presence has been documented in five other northern Great Plains states (Heap 2013). However, evolution, spread, and inheritance of glyphosate resistance in kochia is unknown. An efficient method for determining level of glyphosate resistance in kochia plants may facilitate such studies and the *in vivo* shikimate accumulation test described by Shaner et al. (2005) is one of the potential tools. *In vivo* shikimate accumulation testing with a single glyphosate concentration (100 µM) and incubation time (16 h) discriminated GR kochia populations from the glyphosate-susceptible (GS) population (Chapter 1, Wiersma 2012). However, shikimate accumulation in resistant plants did not correspond well to the EPSPS gene copy number (Wiersma 2012). Increased shikimate accumulation with increasing concentration of glyphosate and incubation time has been reported in other crops and weeds (Culpepper et al. 2006, Shaner et al. 2005, Koger et al. 2005a).

The objectives of this study were to (1) determine the effects of glyphosate concentrations and incubation times on shikimate accumulation in leaf discs of GR and GS kochia plants, and (2) assess if the shikimate accumulation results can be used to predict EPSPS gene copy numbers in kochia plants.

Materials and Methods

Raising Seedlings

Seed of GR and GS kochia populations from western Kansas were sown in 50-cm by 25-cm by 5-cm plastic trays containing 4-cm deep commercial potting mix (Miracle-Gro, Scotts Miracle-Gro Products Inc., Marysville, OH) in greenhouse. When plants were 3 cm tall, 48 plants from the GR and 12 plants from the GS populations were transplanted separately into 12-cm by 12-cm by15-cm plastic pots. Plants were grown under 25/20 (±2) C day/night temperature with 15/9 h day/night photoperiod, supplemented with 200 µmol $m^{-2}s^{-1}$ illumination provided with sodium vapors lamps. Plants were regularly watered as needed and fertilized (Miracle-Gro, Scotts Miracle-Gro Products Inc., Marysville, OH) every two weeks. After analyzing the results of the shikimate accumulation assay with a single concentration of glyphosate (as described below), eight plants from the GR population and three plants from the GS population were transferred to 25-cm diam pots and grown as described previously.

Shikimate Accumulation Assay

Shikimate accumulation assay was conducted using the method described by Shaner et al. (2005) as previously done in Chapter 3. Leaf discs of kochia plants were treated in 200 µM glyphosate solution for 16 h and the accumulated shikimate was measured. Eight plants from the GR population that accumulated <100 ng shikimate mm^{-2} leaf disc (Figure 5.1) and three randomly selected plants from the GS population were selected and transferred into larger pots and grown as described previously. Based on the shikimate accumulation results in previous studies (Chapter 1 and 3), the selected plants from the GR population were assumed to be resistant to glyphosate and hereafter are identified as GR plants. Leaf discs from the selected GR plants (>30-cm tall) were treated in 0, 50, 100, 200, 400, and 800 µM glyphosate solution and the leaf discs from the selected GS plants (>30-cm tall) were treated in 0, 25, 50, 100, 200, and 400 µM glyphosate solution. The leaf discs were incubated for 16 h under continuous light (200 µmol m^{-2}s^{-1}). In another study, the leaf discs were treated with 100 µM solution and incubated for 12, 24, and 48 h under continuous light (200 µmol m^{-2}s^{-1}). Leaf discs were excised from the youngest fully-expanded leaves from each plants. The experiments were done in triplicate and were repeated.

Genomic DNA Extraction and Determination of EPSPS Gene Copy Number

Genomic DNA (gDNA) was extracted from 100 mg of young plant leaves by using the Qiagen Dneasy® Plant Mini Kit following the manufacturer’s protocol (Qiagen Handbooks and Protocols, 2012). The leaves were harvested in 1.5 ml tubes and were immediately frozen in liquid nitrogen. The frozen leaf samples were ground to fine powder in the tubes using a grinding adapter. The extracted gDNA was eluted into 100 µL of Qiagen® AE buffer, and the quality and concentration were determined spectrophotometrically using a Nanodrop® 1000. The DNA samples were stored at -20 C when not in use.

EPSPS gene copy number was determined using quantitative PCR (qPCR) using acetolactate synthase (ALS) gene as a reference gene. The ALS gene copy number is not expected to vary across kochia biotypes (Wiersma 2012) and the copy number is expected to be low (Gaines et. al. 2010). Primers specific to kochia EPSPS and ALS sequence were obtained from Wiersma (2102). The primers were reported to produce 102 and 159 bp EPSPS and ALS products, respectively. The EPSPS forward and reverse primer sequences were 5' GGCCAAAAGGGCAATCGTGGAG 3' and 5' CATTGCCGTTCCCGCGTTTCC 3', respectively. The ALS forward and reverse primer sequences were 5' ATGCAGACAATGTTGGATAC 3' and 5' TCAACCATCGATACGAACAT 3', respectively. Each qPCR reaction (total volume = 20 µL) included 10 uL of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc. Berkeley, CA), 1 µL of each primer (5µM), 2 uL gDNA (8 ng μL^{-1}) template, and 6 µL DI water. Each component of the qPCR reactions was added to the qPCR plates individually. The CFX 96 Real-Time PCR System (Bio-Rad Laboratories, Inc. Berkeley, CA) was used for all qPCR reactions. The initial denaturation step was held at 95 C for 3 min, which was followed by 40 cycles of denaturation at 95 C for 10 sec, and a combined annealing/extension step at 60 C for 30 sec. Each reaction was done in triplicate and the experiment was repeated. The EPSPS:ALS gene copy number was determined using the $2^{\Delta CT}$ method.

Statistical Analysis

Correlation analysis was performed between the amount of shikimate accumulation in leaf discs (for glyphosate concentrations and incubation times) and relative EPSPS:ALS copy number.

Results

Shikimate accumulated in leaf discs of plants from the GR population ranged from 16 to 160 ng shikimate mm^{-2} leaf disc; nearly half of the plants accumulated <100 ng shikimate mm^{-2} leaf disc (Figure 5.1). All plants from the GS population accumulated >100 ng shikimate mm^{-2} leaf disc (data not shown).

The amount of shikimate accumulated in leaf discs increased with increasing glyphosate concentration for all GR and GS plants (Figure 5.2) and response was fairly consistent for most plants across glyphosate concentrations. Most of the GR plants accumulated >20 ng shikimate mm^{-2} leaf disc at 50 µM glyphosate concentration, however, two plants (13R and 30R) accumulated nearly as much shikimate as the GS plants (>60 ng mm^{-2} leaf disc). The difference between GR and GS plants in the amount of shikimate accumulated in leaf discs decreased as glyphosate concentrations increased.

All plants accumulated higher amounts of shikimate at longer incubation times (Figure 5.3). While more than half of the GR plants accumulated >100 ng shikimate mm^{-2} leaf disc, the amount of shikimate accumulation for all GS plants was >200 ng mm^{-2} leaf disc at 24 h incubation time. In general, the amount of shikimate accumulated in leaf discs increased by 2- to 4.5-fold at 48 h compared to the amount of shikimate accumulated at the 24 h incubation time. Most GR plants accumulated 200 to 400 ng shikimate mm^{-2} leaf disc and all GS plants accumulated at least 600 ng shikimate mm^{-2} leaf disc at the 48 h incubation time.

The GR plants had 3.7 to 9.5 copies of relative EPSPS:ALS genes (Figure 5.2). In contrast, EPSPS gene copies relative to ALS ranged from 0.6 to 0.88 for GS plants.

Amount of shikimate accumulated in leaf disc and relative EPSPS:ALS gene copy number correlated negatively across all glyphosate concentrations (Figure 5.4). A stronger correlation of the copy number was observed with 100 and 200 µM glyphosate concentration (R^2=0.86 to 0.88) than with 400 µM concentration (R^2=0.53). Leaf discs of kochia plants with low EPSPS gene copies, accumulated more shikimate. In contrast, leaf discs from plants with high EPSPS gene copies accumulated roughly proportionally less shikimate.

The amount of shikimate accumulated in leaf discs negatively correlated with relative EPSPS:ALS gene copy number at all the incubation times (Figure 5.5) (R^2=0.69 to 0.82). Generally, leaf discs of plants with low EPSPS gene copies accumulated more shikimate than plants with high EPSPS gene copies.

Discussion

More shikimate accumulated in leaf discs of all kochia plants at higher concentrations of glyphosate or longer incubations times. However, a slightly larger difference in the amount of shikimate accumulated was observed at 50 and 100 µM than at 400 and 800 µM glyphosate concentrations. Shikimate accumulated in the leaf discs more by increasing incubation time than by increasing glyphosate concentration. Considerably more shikimate accumulated in GR plants when the leaf discs were incubated for more than 24 h. However, the amount of shikimate accumulated differentiated GR from GS kochia plants at all incubation times.

In general, plants with high EPSPS copies accumulated less shikimate across glyphosate concentrations and incubation times than GS plants or GR plants with low EPSPS copies. This was expected as additional EPSPS genes in kochia plants confer proportionally greater level of resistance to glyphosate (Wiersma 2012). However, some plants with high EPSPS copies consistently accumulated (across glyphosate concentrations and incubation times) similar amounts of shikimate to that of the plants with low EPSPS (Figure 5.4 and 5.5). Variations in leaf morphology and chlorophyll content may affect shikimate accumulation. In addition, photospectrometric shikimate accumulation tests may not be as precise as the HPLC method (Shaner 2010). Thus, in studies that require information on the level of glyphosate resistance in kochia plants, the *in vivo* leaf disc shikimate accumulation test can be more useful in large-scale screening of individuals and/or populations of interest.

References

Alibhai, M. F. and W. C. Stalling. 2001. Closing down on glyphosate inhibition-with a new structure for drug discovery. PNAS 98:2944-2946.

Culpepper, A. S., T. L. Grey, W. K. Vencill, J. M. Kichler, T. M. Webster, S. M. Brown, A. C. York, J. W. Davis, and W. M. Hanna. 2006. Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) confirmed in Georgia. Weed Sci. 54:620-626.

Devine, M. amd A. Shukla. 2000. Altered target sites as a mechanism of herbicide resistance. Crop Prot. 19:881-889.

Funke, T., H. Han, M. L. Healy-Fried, M. Fischer, and E. Schönbrunn. 2006. Molecular basis for the herbicide resistance of Roundup Ready crops. PNAS 103.13010-13015.

Gaines, T. A., W. Zhang, D. Wang, B. Bukun, S. T. Chisholm, D. L. Shaner, S. J. Nissen, W. L. Patzoldt, P. J. Tranel, A. S. Culpepper, T. L. Grey, T. M. Webster, W. K. Vencill, R. D. Sammons, J. Jiang, C. Preston, J. E. Leach, and P. Westra. 2010. Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. PNAS. 107:1029-1034.

Green, J. M. 2009. Evolution of glyphosate-resistant crop technology. Weed Sci. 57:108-117.

Heap, I. The International Survey of Herbicide Resistant Weeds. Online. Internet. Accessed June 29, 2013. Available www.weedscience.com

Henry, W. B., D. L. Shaner, and M. S. West. 2007. Shikimate accumulation in sunflower, wheat, and proso millet after glyphosate application. Weed Sci. 55:1-5.

Herrmann, K. M. and L. M. Weaver. 1999. The shikimate pathway. Annu. Rev. Plant Physiol. Plant. Mol. Biol. 50:473-503.

Koger, C. H., D. L. Shaner, W. B. Henry, T. Nadler-Hassar, W. E. Thomas, and J. W. Wilcut. 2005a. Assessment of two nondestructive assays for detecting glyphosate resistance in horseweed (*Conyza canadensis*). Weed Sci. 53:438-445.

Koger, C. H., D. L. Shaner, L. J. Krutz, T. W. Walker, N. Buehring, W. B. Henry, W. E. Thomas, and J. W. Wilcut. 2005b. Rice (Oryza sativa) response to drift rates of glyphosate. Pest Manag. Sci. 61:1161-1167.

Koger, C. H. and K. N. Reddy. 2005. Role of absorption and trasloction in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). Weed Sci. 53:84-89.

Mueller, T. C., J. H. Massey, R. M. Hayes, C. L. Main, and C. N. Stewart. 2003. Shikimate accumulates in both glyphosate-sensitive and glyphosate-resistant horseweed (*Conyza canadensis* L. Cronq.). J. Agr. Food. Chem. 51:680-684.

Muller, T., A. Tellis, J. E. Beeler, S. D. Sharma, and M. Singh. 2008. Shikimate accumulation in nine weedy species following glyphosate application. Weed Res. 48:455-460.

Perez-Jones, A., K. W. Park, N. Polge, J. Colquhoun, and C. A. Mallory-Smith. 2007. Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. Planta 226:395-404.

Pline-Srnic, W. 2006. Physiological mechanism of glyphosate resistance. Weed Sci. 20:290-300.

Preston, C. and C. A. Mallory-Smith. 2001. Biochemical mechanism, inheritance, and molecular genetics of herbicide resistance in weeds. In S. Powles and D. Shaner, eds. Herbicide resistance and world grains. CRS Press, pp. 23-60.

Preston, C. 2004. Herbicide resistance in weeds endowed by enhanced detoxification: complications for management. Weed Sci. 52:448-453.

Salas, R. A., F. E. Dayan, Z. Pan, S. B. Watson, J. W. Dickson, R. C. Scott, and N. R. Burgos. 2012. EPSPS gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) from Arkansas. Pest Manag. Sci. 68:1223-1230.

Shaner, D. L., T. Nadler-Hasser, and C. H. Koger. 2005. A rapid in vivo shikimate accumulation assay with excised leaf discs. Weed Sci. 53:769-774.

Simarmata, M., J. E. Kaufmann, and D. Penner. 2003. Potential basis of glyphosate resistance in California rigid ryegrass (*Lolium rigidum*). Weed Sci. 51:678-682.

Steinrucken, H .C. and N. Amrbein. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochem. Bioph**.** Res. Co. 94:1207-1212.

Wiersma, A. 2012. Regional whole plant and molecular response of Kochia scoparia to glyphosate. M.S. thesis. Fort Collins, Co. Colorado State University. 63 p.

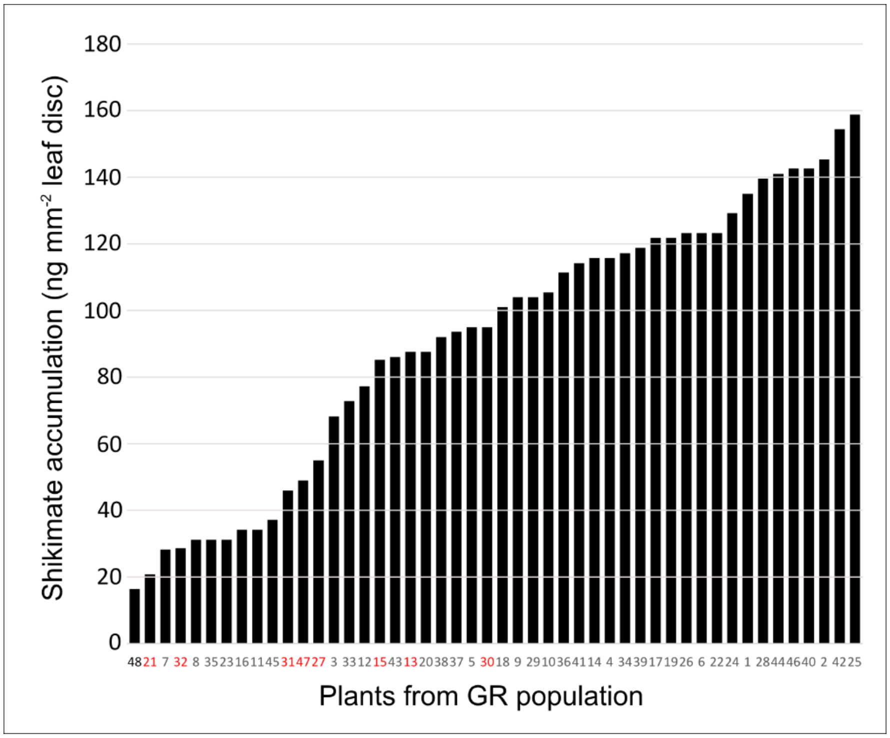


Figure 5.1 Shikimate accumulation in leaf discs of plants from a glyphosate-resistant kochia population. The leaf discs were treated with 100 µM glyphosate for 16 h under continuous light.

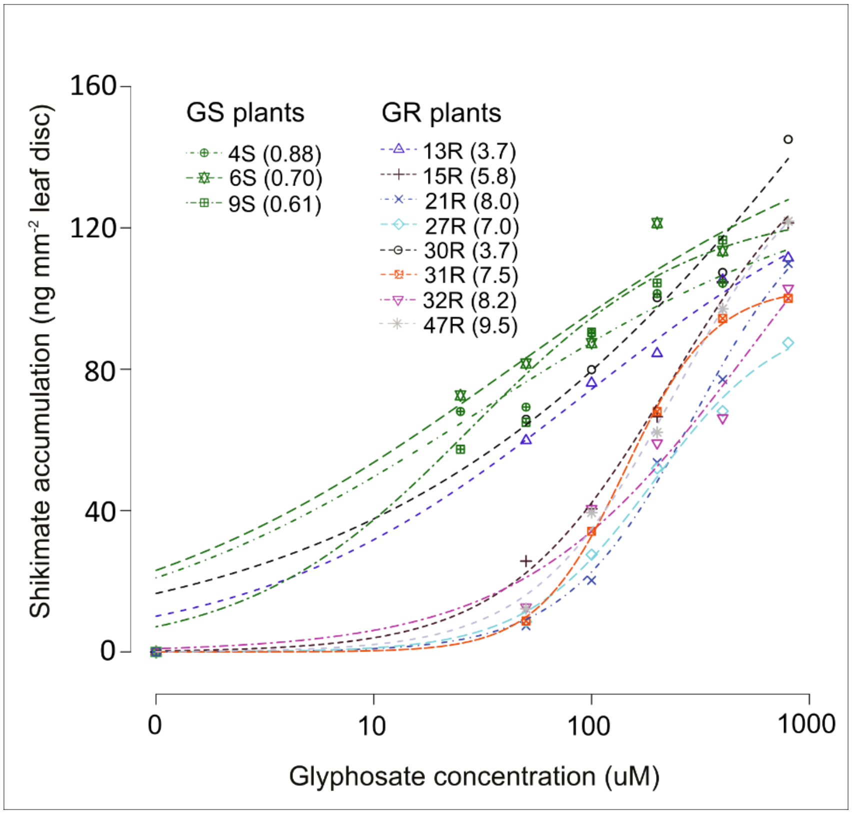


Figure 5.2 Shikimate accumulation in leaf discs of glyphosate-resistant (GR) and glyphosate-susceptible (GS) kochia plants treated with a series of glyphosate concentrations for 16 h under continous light. Values in paranthesis are the relative EPSPS:ALS gene copy number.

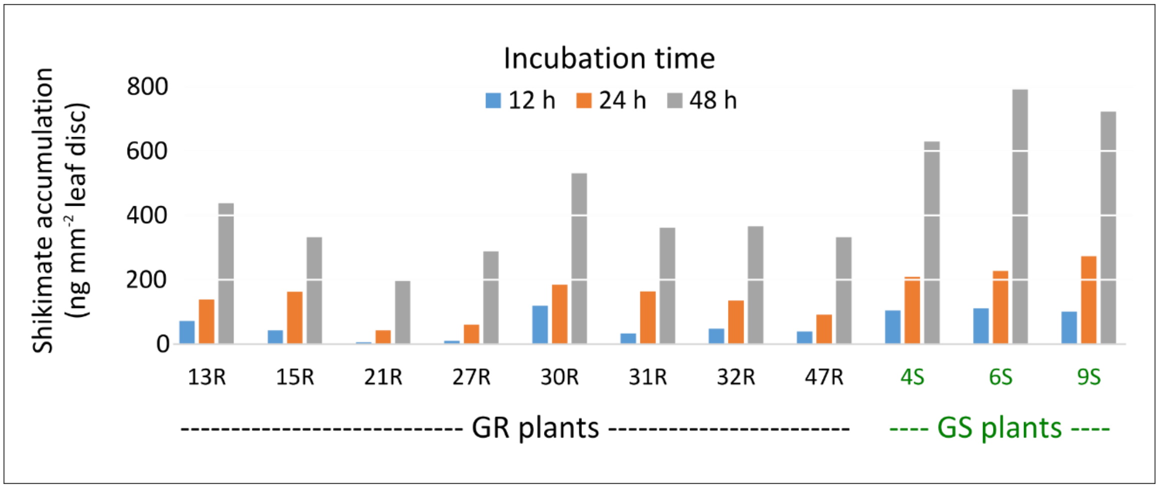


Figure 5.3 Shikimate accumulation in leaf discs of glyphosate-resistant (GR) and glyphosate-susceptible (GS) kochia plants at different incubation times. Leaf disc were treated with 100 µM glyphosate concentration.

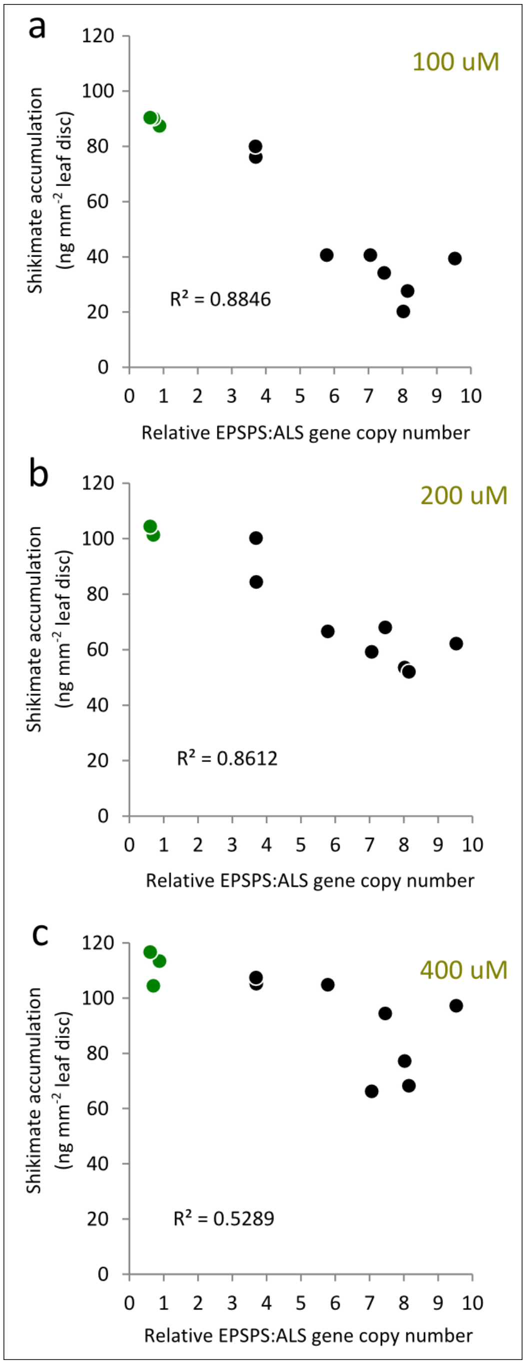


Figure 5.4 Relationship between shikimate accumulation in glyphosate-treated leaf discs and relative EPSPS:ALS genomic copy number in kochia plants. (a) Leaf discs were treated with 100 µM glyphosate solution, (b) Leaf discs were treated with 200 µM glyphosate solution, (c) Leaf discs were treated with 400 µM glyphosate solution.

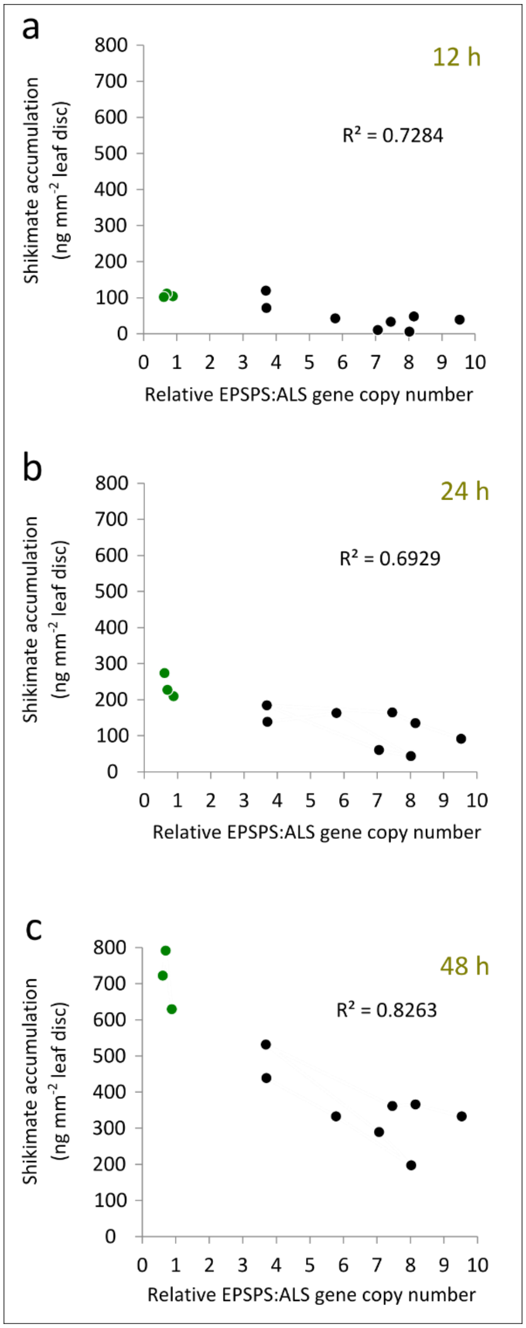
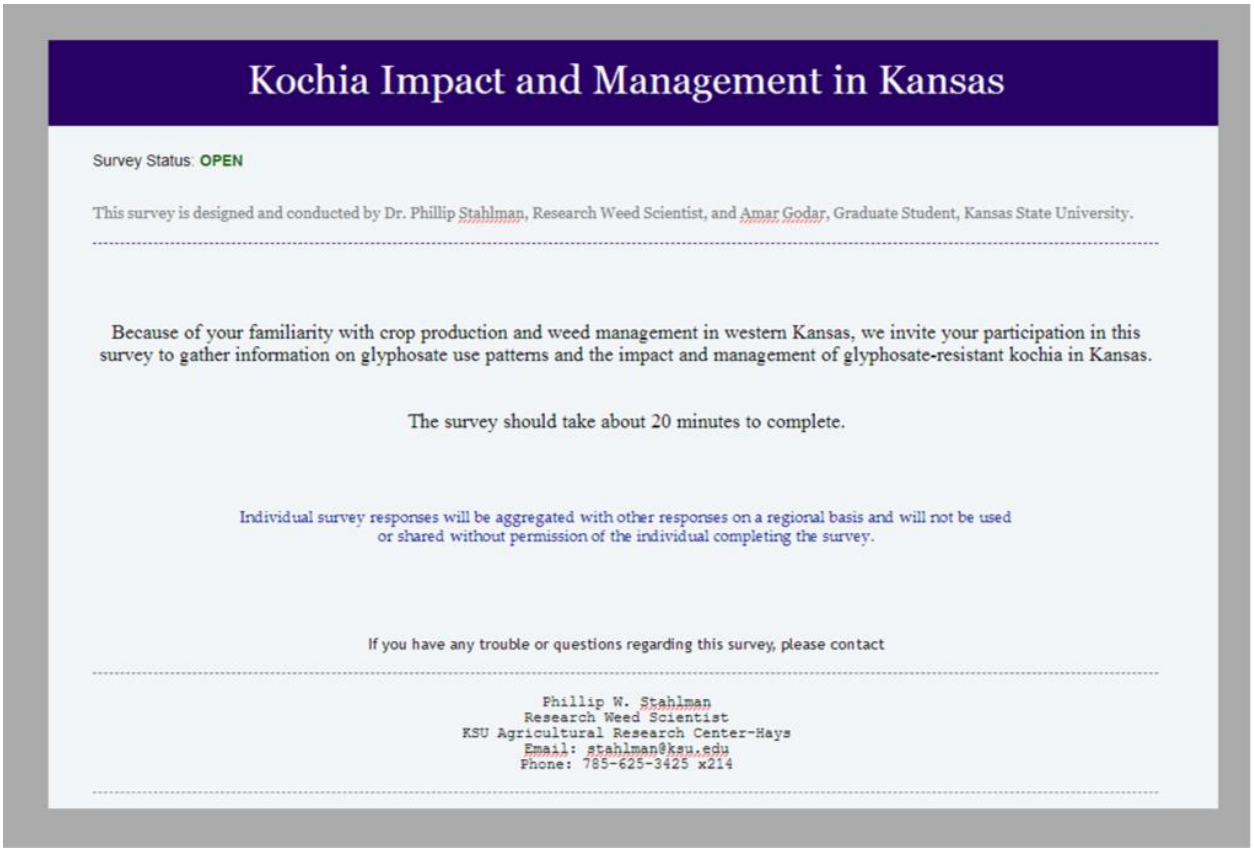


Figure 5.5 Relationship between shikimate accumulation in glyphosate-treated leaf discs and relative EPSPS:ALS genomic copy number in kochia plants. (a) 12 h incubation time, (b) 24 h incubation time, (c) 48 h incubation time.

Appendix – A1: Survey Questionnaire

Snapshots of the survey design in Adobe® Formscentral® is shown below.

Fillable form is available at https://adobeformscentral.com/?f=cep4qWMI9aUYBZUeyWiVRQ



Kochia Impact and Management in Kansas

Survey Status: **OPEN**

This survey is designed and conducted by Dr. Phillip Stahlman, Research Weed Scientist, and Amar Godar, Graduate Student, Kansas State University.

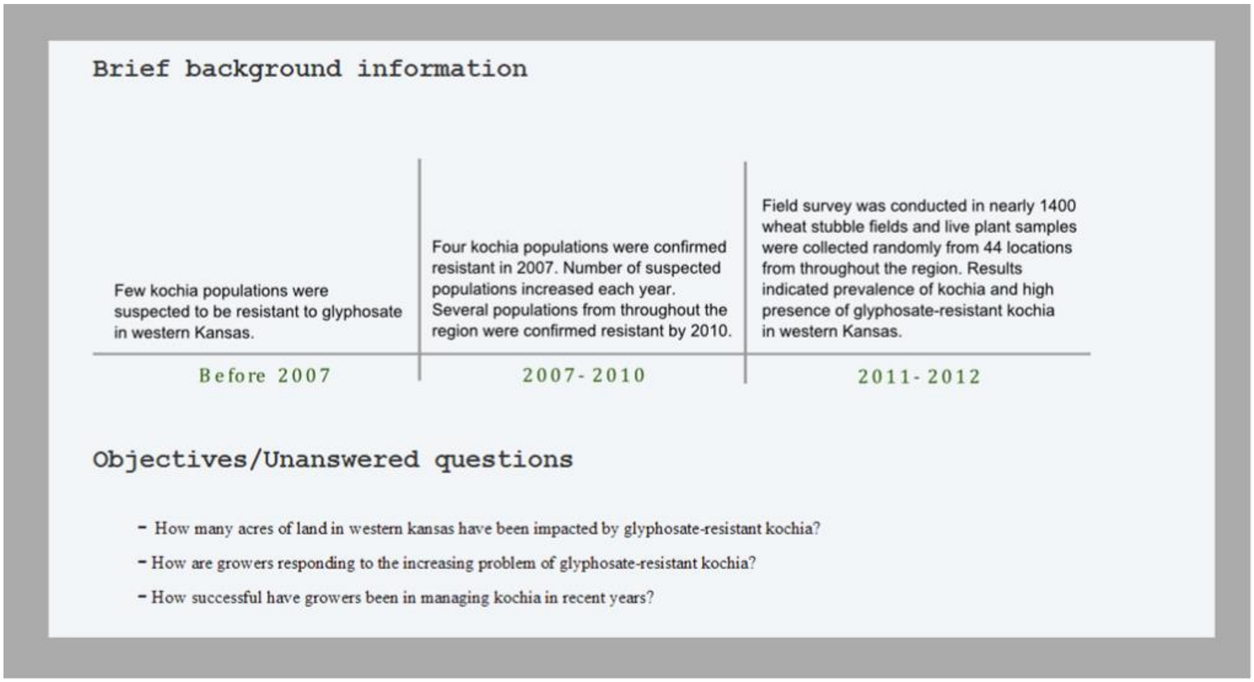
Because of your familiarity with crop production and weed management in western Kansas, we invite your participation in this survey to gather information on glyphosate use patterns and the impact and management of glyphosate-resistant kochia in Kansas.

The survey should take about 20 minutes to complete.

Individual survey responses will be aggregated with other responses on a regional basis and will not be used or shared without permission of the individual completing the survey.

If you have any trouble or questions regarding this survey, please contact

Phillip W. Stahlman
Research Weed Scientist
KSU Agricultural Research Center-Hays
Email: stahlman@ksu.edu
Phone: 785-625-3425 x214



Brief background information

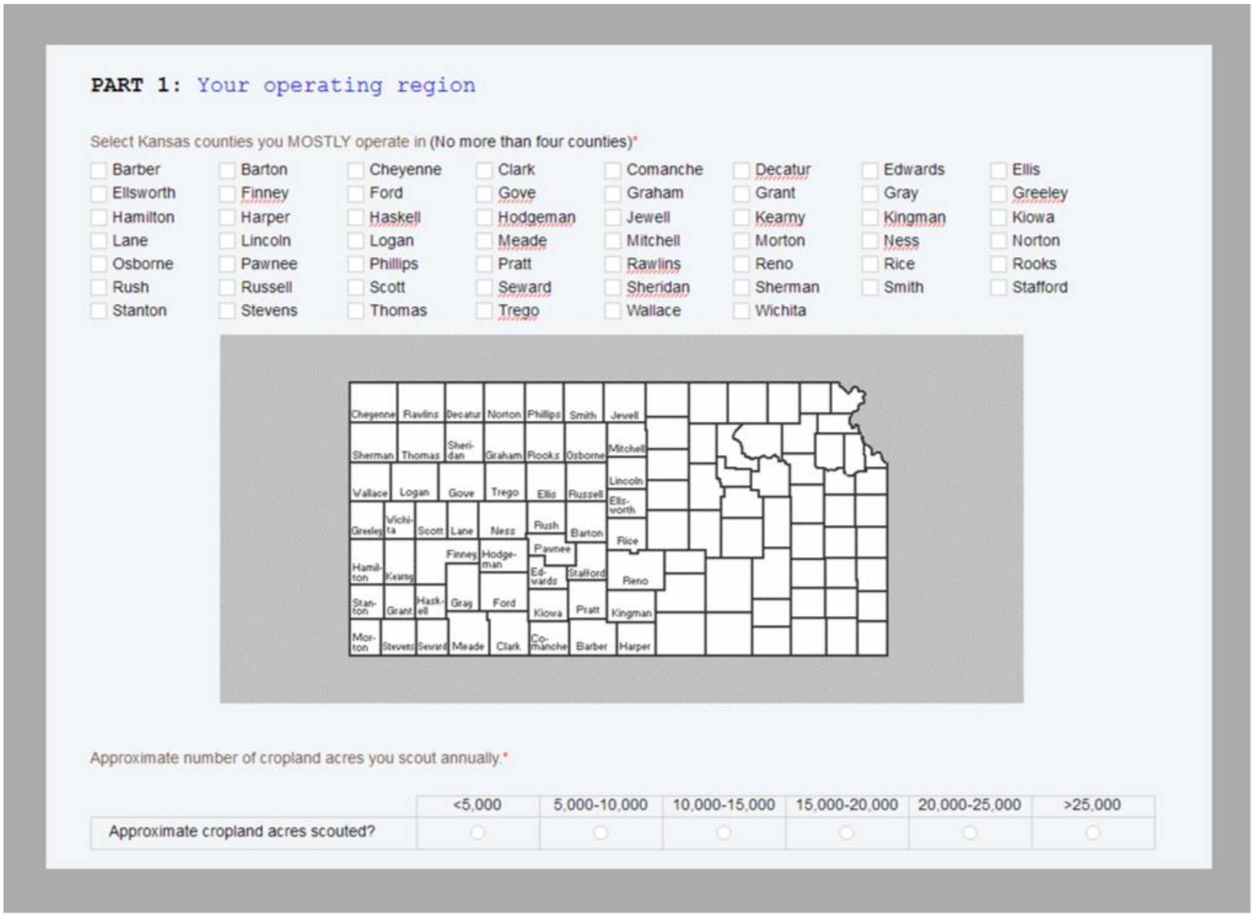
Few kochia populations were suspected to be resistant to glyphosate in western Kansas.

Four kochia populations were confirmed resistant in 2007. Number of suspected populations increased each year. Several populations from throughout the region were confirmed resistant by 2010.

Field survey was conducted in nearly 1400 wheat stubble fields and live plant samples were collected randomly from 44 locations from throughout the region. Results indicated prevalence of kochia and high presence of glyphosate-resistant kochia in western Kansas.

Objectives/Unanswered questions

- How many acres of land in western kansas have been impacted by glyphosate-resistant kochia?
- How are growers responding to the increasing problem of glyphosate-resistant kochia?
- How successful have growers been in managing kochia in recent years?



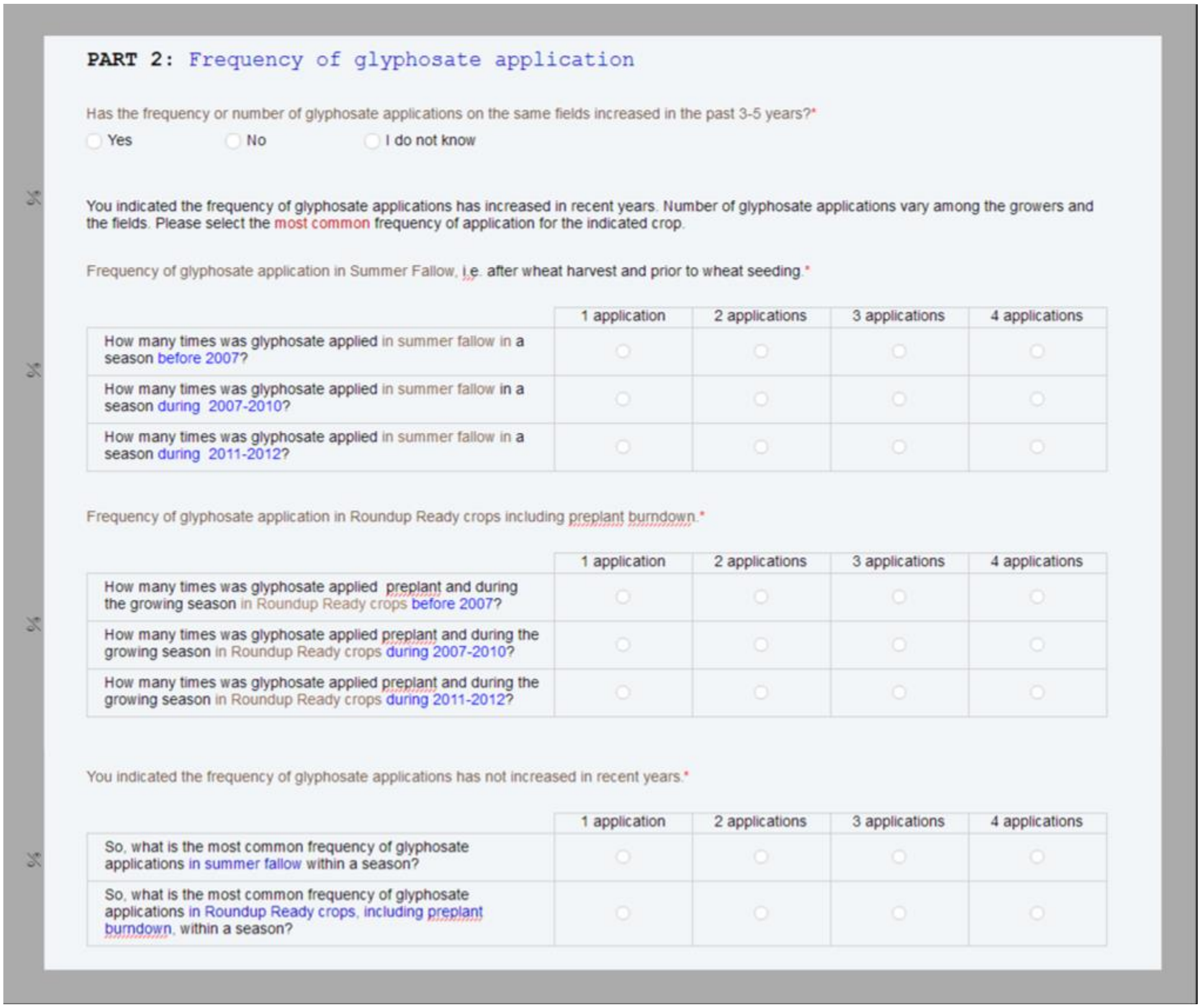
PART 1: Your operating region

Select Kansas counties you MOSTLY operate in (No more than four counties)*

☐ Barber ☐ Barton ☐ Cheyenne ☐ Clark ☐ Comanche ☐ Decatur ☐ Edwards ☐ Ellis
☐ Ellsworth ☐ Finney ☐ Ford ☐ Gove ☐ Graham ☐ Grant ☐ Gray ☐ Greeley
☐ Hamilton ☐ Harper ☐ Haskell ☐ Hodgeman ☐ Jewell ☐ Kearny ☐ Kingman ☐ Kiowa
☐ Lane ☐ Lincoln ☐ Logan ☐ Meade ☐ Mitchell ☐ Morton ☐ Ness ☐ Norton
☐ Osborne ☐ Pawnee ☐ Phillips ☐ Pratt ☐ Rawlins ☐ Reno ☐ Rice ☐ Rooks
☐ Rush ☐ Russell ☐ Scott ☐ Seward ☐ Sheridan ☐ Sherman ☐ Smith ☐ Stafford
☐ Stanton ☐ Stevens ☐ Thomas ☐ Trego ☐ Wallace ☐ Wichita

Approximate number of cropland acres you scout annually.*

	<5,000	5,000-10,000	10,000-15,000	15,000-20,000	20,000-25,000	>25,000
Approximate cropland acres scouted?	○	○	○	○	○	○



PART 2: Frequency of glyphosate application

Has the frequency or number of glyphosate applications on the same fields increased in the past 3-5 years?*

○ Yes ○ No ○ I do not know

You indicated the frequency of glyphosate applications has increased in recent years. Number of glyphosate applications vary among the growers and the fields. Please select the most common frequency of application for the indicated crop.

Frequency of glyphosate application in Summer Fallow, i.e. after wheat harvest and prior to wheat seeding.*

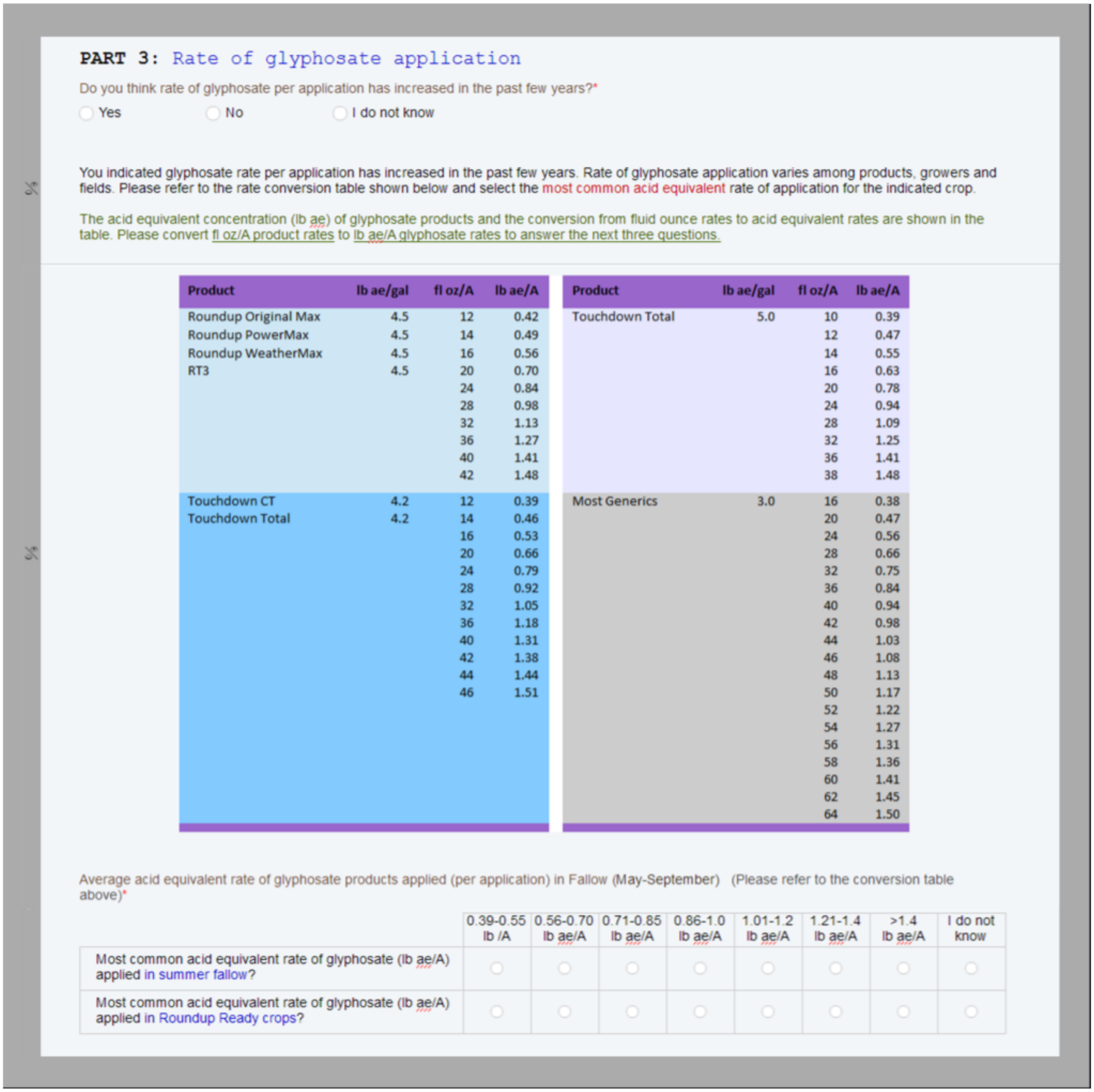
	1 application	2 applications	3 applications	4 applications
How many times was glyphosate applied in summer fallow in a season before 2007?	○	○	○	○
How many times was glyphosate applied in summer fallow in a season during 2007-2010?	○	○	○	○
How many times was glyphosate applied in summer fallow in a season during 2011-2012?	○	○	○	○

Frequency of glyphosate application in Roundup Ready crops including preplant burndown.*

	1 application	2 applications	3 applications	4 applications
How many times was glyphosate applied preplant and during the growing season in Roundup Ready crops before 2007?	○	○	○	○
How many times was glyphosate applied preplant and during the growing season in Roundup Ready crops during 2007-2010?	○	○	○	○
How many times was glyphosate applied preplant and during the growing season in Roundup Ready crops during 2011-2012?	○	○	○	○

You indicated the frequency of glyphosate applications has not increased in recent years.*

	1 application	2 applications	3 applications	4 applications
So, what is the most common frequency of glyphosate applications in summer fallow within a season?	○	○	○	○
So, what is the most common frequency of glyphosate applications in Roundup Ready crops, including preplant burndown, within a season?	○	○	○	○



PART 3: Rate of glyphosate application

Do you think rate of glyphosate per application has increased in the past few years?*

○ Yes ○ No ○ I do not know

You indicated glyphosate rate per application has increased in the past few years. Rate of glyphosate application varies among products, growers and fields. Please refer to the rate conversion table shown below and select the most common acid equivalent rate of application for the indicated crop.

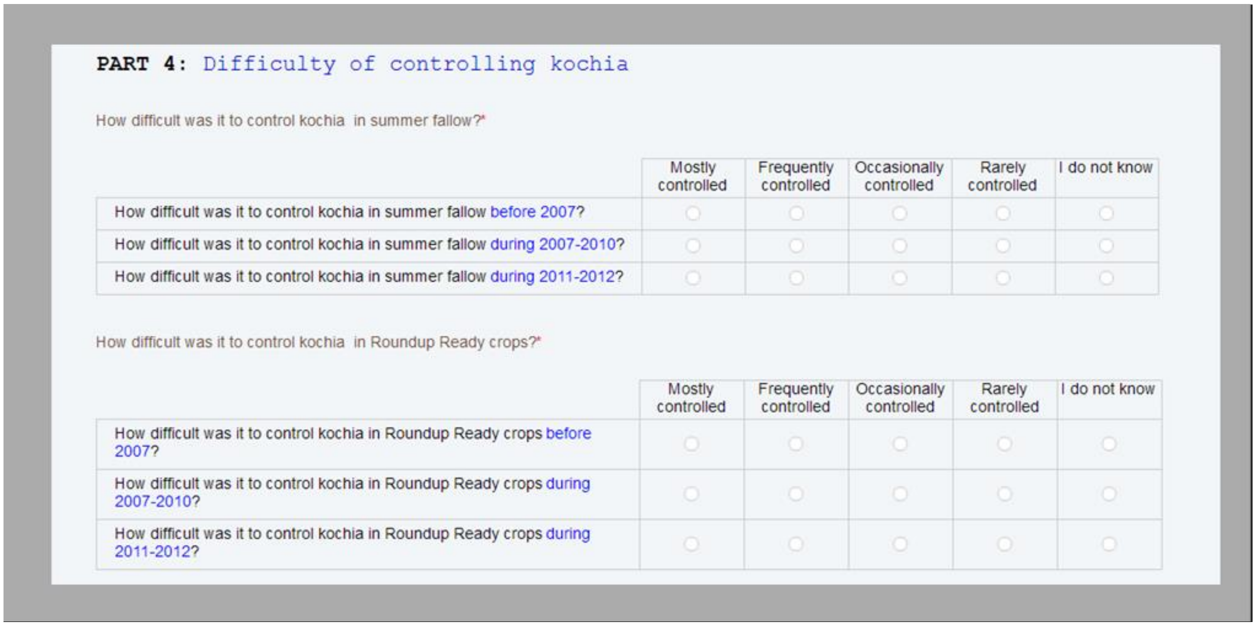
The acid equivalent concentration (lb ae) of glyphosate products and the conversion from fluid ounce rates to acid equivalent rates are shown in the table. Please convert fl oz/A product rates to lb ae/A glyphosate rates to answer the next three questions.

Product	lb ae/gal	fl oz/A	lb ae/A
Roundup Original Max	4.5	12	0.42
Roundup PowerMax	4.5	14	0.49
Roundup WeatherMax	4.5	16	0.56
RT3	4.5	20	0.70
		24	0.84
		28	0.98
		32	1.13
		36	1.27
		40	1.41
		42	1.48
Touchdown CT	4.2	12	0.39
Touchdown Total	4.2	14	0.46
		16	0.53
		20	0.66
		24	0.79
		28	0.92
		32	1.05
		36	1.18
		40	1.31
		42	1.38
		44	1.44
		46	1.51

Product	lb ae/gal	fl oz/A	lb ae/A
Touchdown Total	5.0	10	0.39
		12	0.47
		14	0.55
		16	0.63
		20	0.78
		24	0.94
		28	1.09
		32	1.25
		36	1.41
		38	1.48
Most Generics	3.0	16	0.38
		20	0.47
		24	0.56
		28	0.66
		32	0.75
		36	0.84
		40	0.94
		42	0.98
		44	1.03
		46	1.08
		48	1.13
		50	1.17
		52	1.22
		54	1.27
		56	1.31
		58	1.36
		60	1.41
		62	1.45
		64	1.50

Average acid equivalent rate of glyphosate products applied (per application) in Fallow (May-September) (Please refer to the conversion table above)*

	0.39-0.55 lb /A	0.56-0.70 lb ae/A	0.71-0.85 lb ae/A	0.86-1.0 lb ae/A	1.01-1.2 lb ae/A	1.21-1.4 lb ae/A	>1.4 lb ae/A	I do not know
Most common acid equivalent rate of glyphosate (lb ae/A) applied in summer fallow?	○	○	○	○	○	○	○	○
Most common acid equivalent rate of glyphosate (lb ae/A) applied in Roundup Ready crops?	○	○	○	○	○	○	○	○



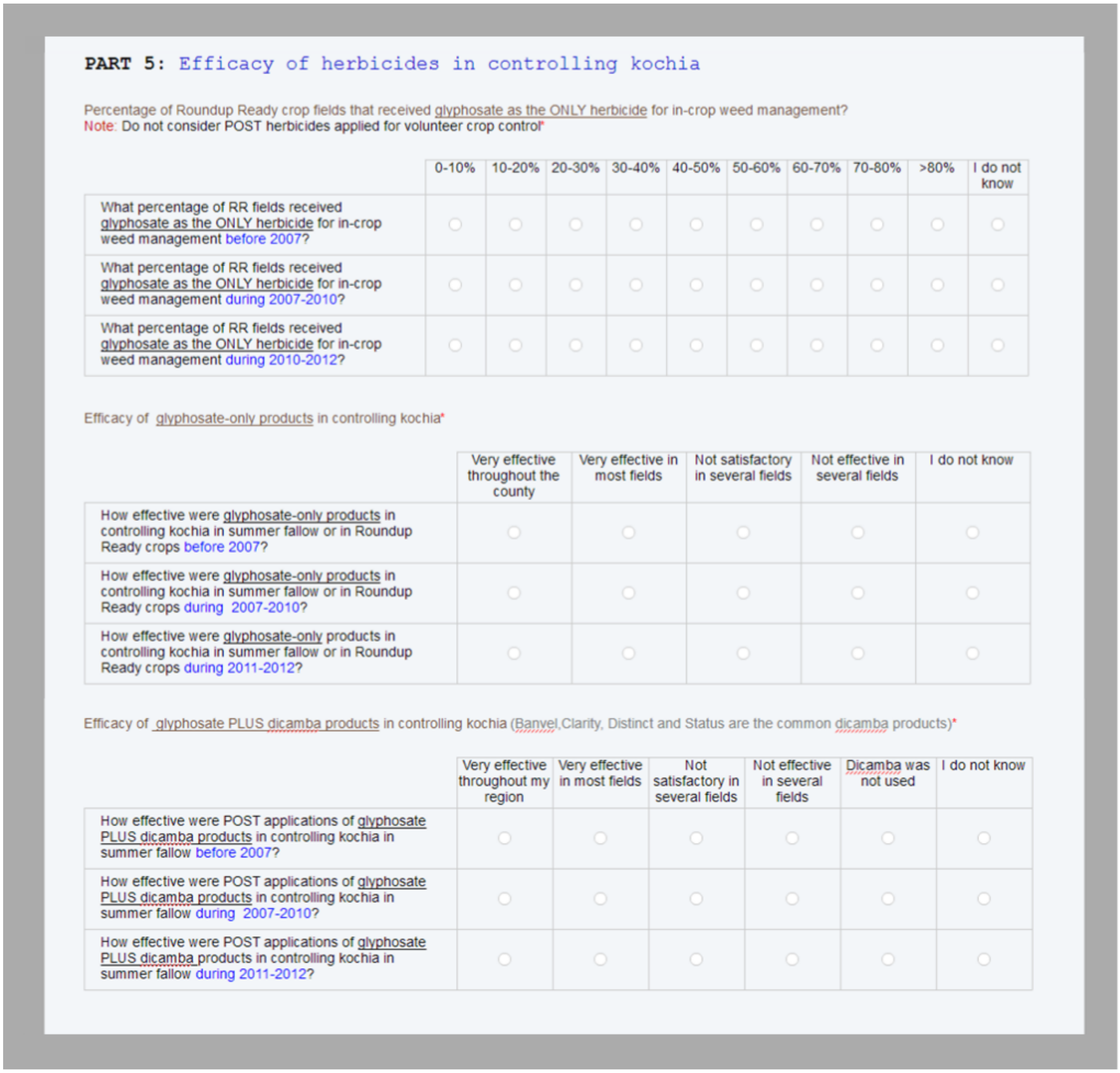
PART 4: Difficulty of controlling kochia

How difficult was it to control kochia in summer fallow?*

	Mostly controlled	Frequently controlled	Occasionally controlled	Rarely controlled	I do not know
How difficult was it to control kochia in summer fallow before 2007?	○	○	○	○	○
How difficult was it to control kochia in summer fallow during 2007-2010?	○	○	○	○	○
How difficult was it to control kochia in summer fallow during 2011-2012?	○	○	○	○	○

How difficult was it to control kochia in Roundup Ready crops?*

	Mostly controlled	Frequently controlled	Occasionally controlled	Rarely controlled	I do not know
How difficult was it to control kochia in Roundup Ready crops before 2007?	○	○	○	○	○
How difficult was it to control kochia in Roundup Ready crops during 2007-2010?	○	○	○	○	○
How difficult was it to control kochia in Roundup Ready crops during 2011-2012?	○	○	○	○	○



PART 5: Efficacy of herbicides in controlling kochia

Percentage of Roundup Ready crop fields that received glyphosate as the ONLY herbicide for in-crop weed management?
Note: Do not consider POST herbicides applied for volunteer crop control*

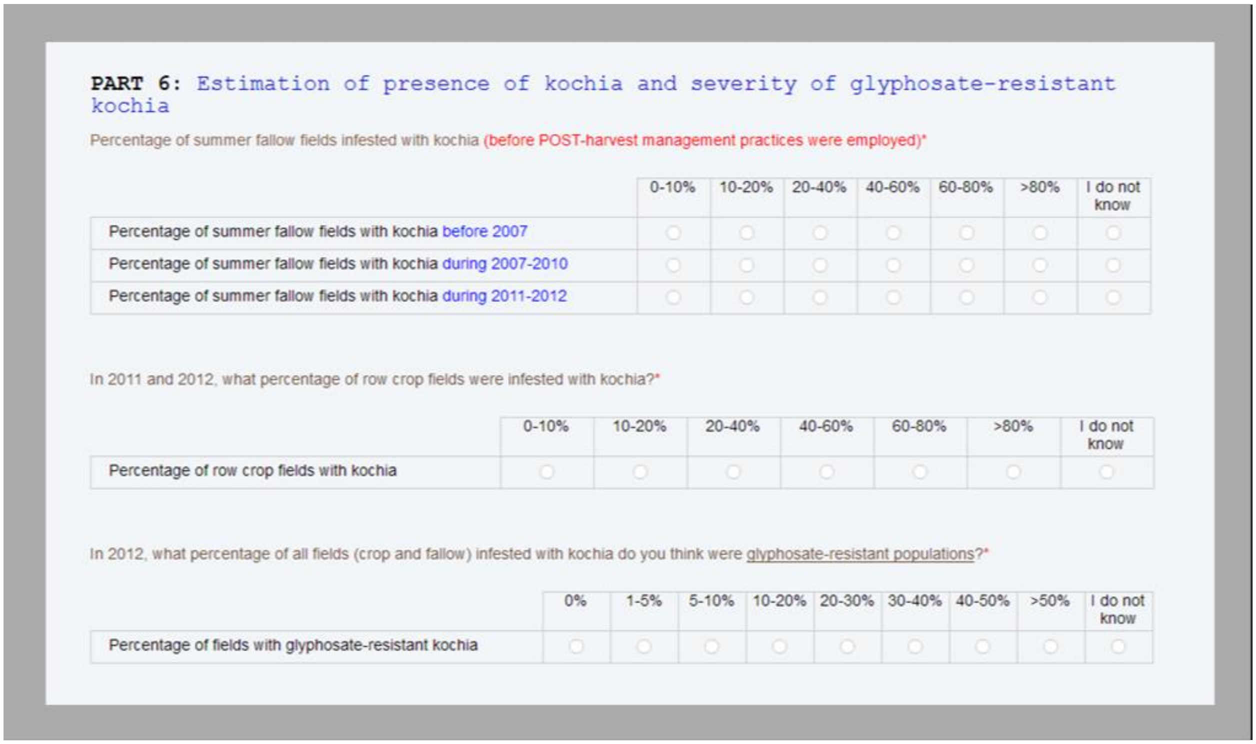
	0-10%	10-20%	20-30%	30-40%	40-50%	50-60%	60-70%	70-80%	>80%	I do not know
What percentage of RR fields received glyphosate as the ONLY herbicide for in-crop weed management before 2007?	○	○	○	○	○	○	○	○	○	○
What percentage of RR fields received glyphosate as the ONLY herbicide for in-crop weed management during 2007-2010?	○	○	○	○	○	○	○	○	○	○
What percentage of RR fields received glyphosate as the ONLY herbicide for in-crop weed management during 2010-2012?	○	○	○	○	○	○	○	○	○	○

Efficacy of glyphosate-only products in controlling kochia*

	Very effective throughout the county	Very effective in most fields	Not satisfactory in several fields	Not effective in several fields	I do not know
How effective were glyphosate-only products in controlling kochia in summer fallow or in Roundup Ready crops before 2007?	○	○	○	○	○
How effective were glyphosate-only products in controlling kochia in summer fallow or in Roundup Ready crops during 2007-2010?	○	○	○	○	○
How effective were glyphosate-only products in controlling kochia in summer fallow or in Roundup Ready crops during 2011-2012?	○	○	○	○	○

Efficacy of glyphosate PLUS dicamba products in controlling kochia (Banvel,Clarity, Distinct and Status are the common dicamba products)*

	Very effective throughout my region	Very effective in most fields	Not satisfactory in several fields	Not effective in several fields	Dicamba was not used	I do not know
How effective were POST applications of glyphosate PLUS dicamba products in controlling kochia in summer fallow before 2007?	○	○	○	○	○	○
How effective were POST applications of glyphosate PLUS dicamba products in controlling kochia in summer fallow during 2007-2010?	○	○	○	○	○	○
How effective were POST applications of glyphosate PLUS dicamba products in controlling kochia in summer fallow during 2011-2012?	○	○	○	○	○	○



PART 6: Estimation of presence of kochia and severity of glyphosate-resistant kochia

Percentage of summer fallow fields infested with kochia (before POST-harvest management practices were employed)*

	0-10%	10-20%	20-40%	40-60%	60-80%	>80%	I do not know
Percentage of summer fallow fields with kochia before 2007	○	○	○	○	○	○	○
Percentage of summer fallow fields with kochia during 2007-2010	○	○	○	○	○	○	○
Percentage of summer fallow fields with kochia during 2011-2012	○	○	○	○	○	○	○

In 2011 and 2012, what percentage of row crop fields were infested with kochia?*

	0-10%	10-20%	20-40%	40-60%	60-80%	>80%	I do not know
Percentage of row crop fields with kochia	○	○	○	○	○	○	○

In 2012, what percentage of all fields (crop and fallow) infested with kochia do you think were glyphosate-resistant populations?*

	0%	1-5%	5-10%	10-20%	20-30%	30-40%	40-50%	>50%	I do not know
Percentage of fields with glyphosate-resistant kochia	○	○	○	○	○	○	○	○	○

PART 7: Management of kochia

Management program targeting kochia control?*

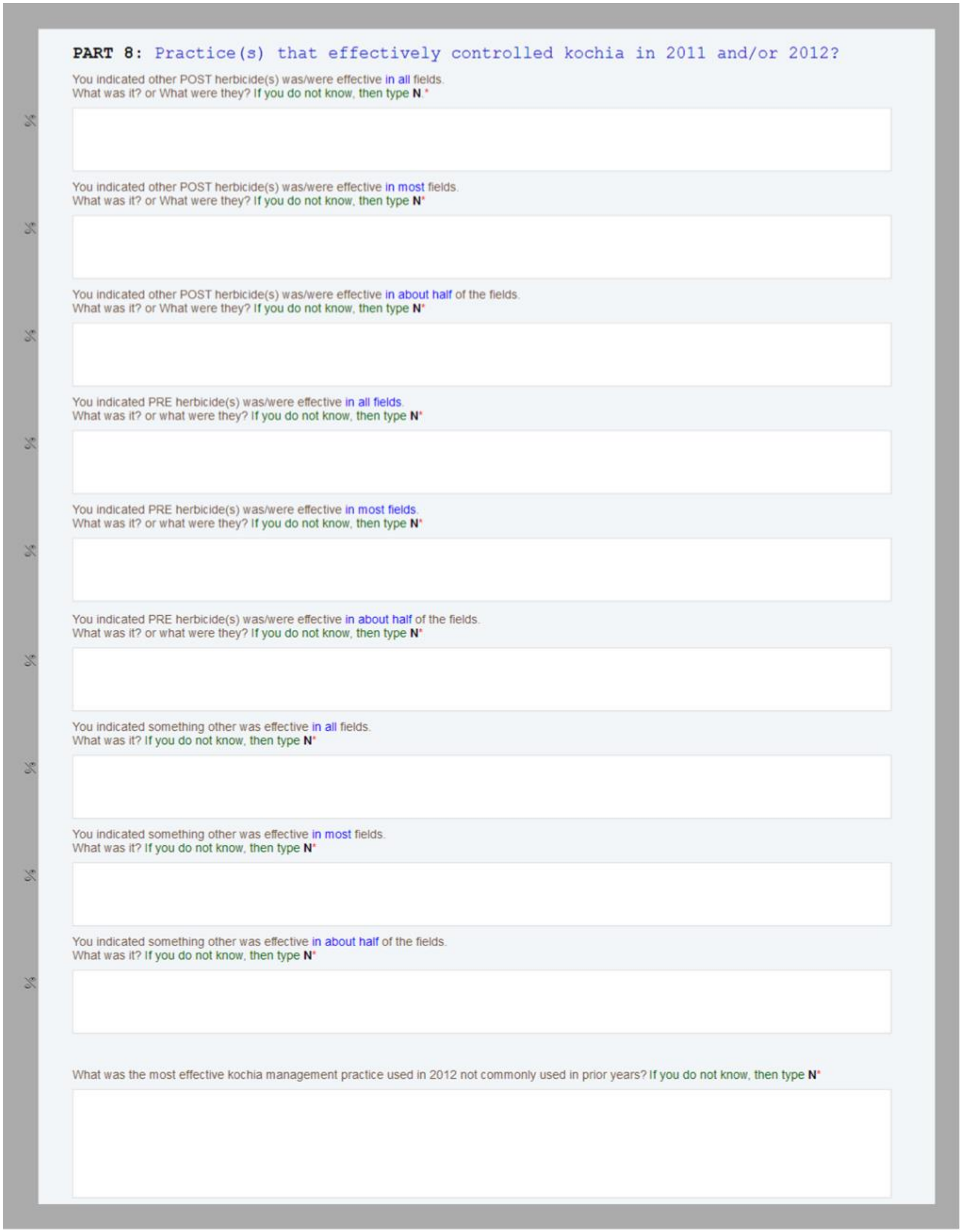
	Never	Sometimes	Occasionally	Always	I do not know
How often did growers' weed management program specifically target kochia control before 2007?	○	○	○	○	○
How often did growers' weed management program specifically target kochia control during 2007-2010?	○	○	○	○	○
How often did growers' weed management program specifically target kochia control during 2011-2102?	○	○	○	○	○

What percentage of your growers implemented the following practices to control kochia in 2011 and/or 2102?*

	0-5%	5-10%	10-20%	20-40%	40-60%	>60%	I do not know
Applied normal rate of glyphosate	○	○	○	○	○	○	○
Applied higher-than-normal rate of glyphosate	○	○	○	○	○	○	○
Applied glyphosate multiple times	○	○	○	○	○	○	○
Tank mixed >8 oz of dicamba product with glyphosate	○	○	○	○	○	○	○
Used other POST herbicides	○	○	○	○	○	○	○
Used PRE herbicides	○	○	○	○	○	○	○
Tilled the field	○	○	○	○	○	○	○
Did something different	○	○	○	○	○	○	○

How do you rate those practices based on their effectiveness in controlling kochia in 2011 and/or 2102?*

	Effective in all fields	Effective in most fields	Effective in about half of fields	Effective in only few fields	Did not work at all	I do not know
Normal rates of glyphosate (0.75-1.0 lb ae/A)	○	○	○	○	○	○
More than 1.0 lb ae/A of glyphosate	○	○	○	○	○	○
Multiple glyphosate applications, normal/higher rates	○	○	○	○	○	○
Tank mixing >8 oz of dicamba product with glyphosate	○	○	○	○	○	○
Other POST herbicide(s)	○	○	○	○	○	○
PRE herbicide(s)	○	○	○	○	○	○
Tillage	○	○	○	○	○	○
Something other	○	○	○	○	○	○



PART 8: Practice(s) that effectively controlled kochia in 2011 and/or 2012?

You indicated other POST herbicide(s) was/were effective in all fields.
What was it? or What were they? If you do not know, then type **N**.*

You indicated other POST herbicide(s) was/were effective in most fields.
What was it? or What were they? If you do not know, then type **N***

You indicated other POST herbicide(s) was/were effective in about half of the fields.
What was it? or What were they? If you do not know, then type **N***

You indicated PRE herbicide(s) was/were effective in all fields.
What was it? or what were they? If you do not know, then type **N***

You indicated PRE herbicide(s) was/were effective in most fields.
What was it? or what were they? If you do not know, then type **N***

You indicated PRE herbicide(s) was/were effective in about half of the fields.
What was it? or what were they? If you do not know, then type **N***

You indicated something other was effective in all fields.
What was it? If you do not know, then type **N***

You indicated something other was effective in most fields.
What was it? If you do not know, then type **N***

You indicated something other was effective in about half of the fields.
What was it? If you do not know, then type **N***

What was the most effective kochia management practice used in 2012 not commonly used in prior years? If you do not know, then type **N***